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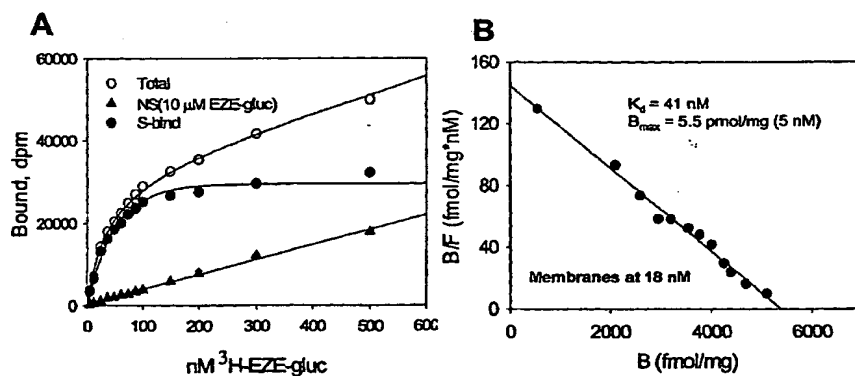
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(54) Title: NPC1L1 (NPC3) AND METHODS OF IDENTIFYING LIGANDS THEREOF



Equilibrium binding of EZE-glucuronide to rhesus BBMVs

(57) Abstract: The present invention provides human, rat and mouse NPC1L1 polypeptides and polynucleotides encoding the polypeptides. Methods for detecting ligands which bind to NPC1L1 and block intestinal cholesterol absorption are provided. Also included is a method of identifying ligands which bind to NPC1L1 using membranes derived from brush border membrane preparations. Compounds that bind to NPC1L1 can be used for inhibiting intestinal cholesterol absorption in a subject.

## **NPC1L1 (NPC3) AND METHODS OF IDENTIFYING LIGANDS THEREOF**

### **SPECIFICATION**

5           The invention claimed herein was made on behalf of Merck & Co., Inc. and Schering-Plough Corporation, parties to a joint research agreement that was in effect on or before the date the claimed invention was made.

          This application claims priority to Serial No. 60/537,341, filed January 16, 2004.

### **FIELD OF THE INVENTION**

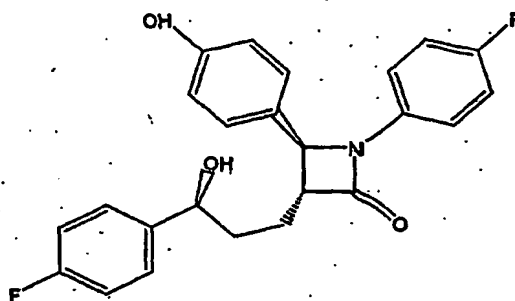
10           The present invention includes NPC1L1 polypeptides and polynucleotides which encode the polypeptides, methods of use and methods of identifying modulators and ligands thereof.

### **BACKGROUND OF THE INVENTION**

15           A factor leading to development of vascular disease, a leading cause of death in industrialized nations, is elevated serum cholesterol. It is estimated that 19% of Americans between the ages of 20 and 74 years of age have high serum cholesterol. The most prevalent form of vascular disease is arteriosclerosis, a condition associated with the thickening and hardening of the arterial wall. Arteriosclerosis of the large  
20   vessels is referred to as atherosclerosis. Atherosclerosis is the predominant underlying factor in vascular disorders such as coronary artery disease, aortic aneurysm, arterial disease of the lower extremities and cerebrovascular disease.

          Cholesteryl esters are a major component of atherosclerotic lesions and the major storage form of cholesterol in arterial wall cells. Formation of cholesteryl  
25   esters is also a step in the intestinal absorption of dietary cholesterol. Thus, inhibition of cholesteryl ester formation and reduction of serum cholesterol can inhibit the progression of atherosclerotic lesion formation, decrease the accumulation of cholesteryl esters in the arterial wall, and block the intestinal absorption of dietary cholesterol.

The regulation of whole-body cholesterol homeostasis in mammals and animals involves the regulation of intestinal cholesterol absorption, cellular cholesterol trafficking, dietary cholesterol and modulation of cholesterol biosynthesis, bile acid biosynthesis, steroid biosynthesis and the catabolism of the cholesterol-containing plasma lipoproteins. Regulation of intestinal cholesterol absorption has proven to be an effective means by which to regulate serum cholesterol levels. For example, a cholesterol absorption inhibitor, ezetimibe (



), has been shown to be effective in this regard. A pharmaceutical composition containing ezetimibe is commercially available from Merck/Schering-Plough Pharmaceuticals, Inc. under the trade name Zetia®. Identification of a gene target through which ezetimibe acts is important to understanding the process of cholesterol absorption and to the development of other, novel absorption inhibitors. The present invention addresses this need by providing a rat and a mouse homologue of human NPC1L1 (also known as NPC3; Genbank Accession No. AF192522; Davies, *et al.*, (2000) Genomics 65(2): 137-45 and Ioannou, (2000) Mol. Genet. Metab. 71(1-2): 175-81), an ezetimibe target.

NPC1L1 is an N-glycosylated protein comprising a YQRL (SEQ ID NO: 38) motif (i.e., a *trans*-golgi network to plasma membrane transport signal; see Bos, *et al.*, (1993) EMBO J. 12: 2219-2228; Humphrey, *et al.*, (1993) J. Cell. Biol. 120: 1123-1135; Ponnambalam, *et al.*, (1994) J. Cell. Biol. 125: 253-268 and Rothman, *et al.*, (1996) Science 272: 227-234) which exhibits limited tissue distribution and gastrointestinal abundance. Also, the human *NPC1L1* promoter includes a Sterol Regulated Element Binding Protein 1 (SREBP1) binding consensus sequence (Athanikar, *et al.*, (1998) Proc. Natl. Acad. Sci. USA 95: 4935-4940; Ericsson, *et al.*, (1996) Proc. Natl. Acad. Sci. USA 93: 945-950; Metherall, *et al.*, (1989) J. Biol. Chem. 264: 15634-15641; Smith, *et al.*, (1990) J. Biol. Chem. 265:

2306-2310; Bennett, *et al.*, (1999) *J. Biol. Chem.* 274: 13025-13032 and Brown, *et al.*, (1997) *Cell* 89: 331-340). NPC1L1 has 42% amino acid sequence homology to human NPC1 (Genbank Accession No. AF002020), a receptor responsible for Niemann-Pick C1 disease (Carstea, *et al.*, (1997) *Science* 277: 228-231). Niemann-Pick C1 disease is a rare genetic disorder in humans which results in accumulation of low density lipoprotein (LDL)-derived unesterified cholesterol in lysosomes (Pentchev, *et al.*, (1994) *Biochim. Biophys. Acta.* 1225: 235-243 and Vanier, *et al.*, (1991) *Biochim. Biophys. Acta.* 1096: 328-337). In addition, cholesterol accumulates in the *trans*-golgi network of npc1<sup>-</sup> cells, and relocation of cholesterol, to and from the plasma membrane, is delayed. NPC1 and NPC1L1 each possess 13 transmembrane spanning segments as well as a sterol-sensing domain (SSD). Several other proteins, including HMG-CoA Reductase (HMG-R), Patched (PTC) and Sterol Regulatory Element Binding Protein Cleavage-Activation Protein (SCAP), include an SSD which is involved in sensing cholesterol levels possibly by a mechanism which involves direct cholesterol binding (Gil, *et al.*, (1985) *Cell* 41: 249-258; Kumagai, *et al.*, (1995) *J. Biol. Chem.* 270: 19107-19113; Hua, *et al.*, (1996) *Cell* 87: 415-426; and Radhakrishnan, A., *et al.*, "Direct binding of cholesterol to the purified membrane region of SCAP: Mechanism for a sterol-sensing domain," *Mol. Cell* 15, 259-268 (2004)).

## SUMMARY OF THE INVENTION

The present invention is based on the discovery that NPC1L1 is the target through which ezetimibe acts, and consequently plays a critical role in the regulation of sterol and 5 $\alpha$ -stanol intestinal transport and absorption, *e.g.* cholesterol absorption. Accordingly, this invention provides for the use of NPC1L1 in an assay for identifying ligands that block NPC1L1-mediated sterol and 5 $\alpha$ -stanol intestinal transport. The present invention provides methods for identifying ligands of NPC1L1 which involve contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and a candidate compound, and determining whether the candidate compound binds to NPC1L1. The modulation of the binding of the substituted 2-azetidinone to NPC1L1 by the binding of the candidate compound to NPC1L1 indicates that the candidate compound is a ligand that binds to NPC1L1 and is an inhibitor of sterol and 5 $\alpha$ -stanol absorption.

The present invention also provides a method for identifying a ligand of NPC1L1 comprising contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and measuring the binding of detectably labeled substituted 2-azetidinone to NPC1L1 in the presence and absence of a candidate compound, wherein decreased binding of the detectably labeled substituted 2-azetidinone to the NPC1L1 in the presence of the candidate compound indicates that said candidate compound is a ligand of NPC1L1 and is an inhibitor of sterol and 5 $\alpha$ -stanol absorption.

The present invention also provides for a method for identifying a compound that inhibits intestinal sterol or 5 $\alpha$ -stanol absorption mediated by NPC1L1 involving contacting NPC1L1 with a detectably labeled ligand and the candidate compound and determining whether the candidate compound binds to NPC1L1, wherein binding of said candidate compound to NPC1L1 modulates binding of said ligand to NPC1L1, wherein said modulation indicates that the candidate compound is an intestinal sterol or 5 $\alpha$ -stanol absorption inhibitor.

The present invention provides methods for identifying an ligand of NPC1L1 comprising (a) contacting a host cell (*e.g.*, human embryonic kidney (HEK) 293 cells, chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface, in the presence of a known amount of a detectably labeled (*e.g.*, with <sup>3</sup>H, <sup>14</sup>C, <sup>125</sup>I, <sup>35</sup>S or fluorescence labeling) substituted azetidinone (*e.g.*, ezetimibe), with a sample to be tested for the presence of an NPC1L1 ligand; and (b) measuring the amount of detectably labeled substituted azetidinone (*e.g.*, ezetimibe) specifically bound to the polypeptide; wherein an NPC1L1 ligand in the sample is identified by measuring substantially reduced binding of the detectably labeled substituted azetidinone (*e.g.*, ezetimibe) to the polypeptide, compared to what would be measured in the absence of such a ligand.

Another method for identifying an ligand of NPC1L1 is also provided. The method comprises (a) placing, in an aqueous suspension, a plurality of support particles, impregnated with a fluorescer (*e.g.*, yttrium silicate, yttrium oxide, diphenyloxazole and polyvinyltoluene), to which a host cell (*e.g.*, chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a

polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface are attached; (b) adding, to the suspension, a radiolabeled (*e.g.*, with  $^3\text{H}$ ,  $^{14}\text{C}$  or  $^{125}\text{I}$ ) substituted azetidinone (*e.g.*, ezetimibe) and a sample to be tested for the presence of a ligand, wherein the radiolabel emits radiation energy capable of activating the fluorescer upon the binding of the substituted azetidinone (*e.g.*, ezetimibe) to the polypeptide to produce light energy, whereas radiolabeled substituted azetidinone (*e.g.*, ezetimibe) that does not bind to the polypeptide is, generally, too far removed from the support particles to enable the radioactive energy to activate the fluorescer; and (c) measuring the light energy emitted by the fluorescer in the suspension; wherein an NPC1L1 ligand in the sample is identified by measuring substantially reduced light energy emission, compared to what would be measured in the absence of such a ligand.

Also provided is a method for identifying a ligand of NPC1L1 comprising (a) contacting a host cell (*e.g.*, Chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface with detectably labeled (*e.g.*, with  $^3\text{H}$ ,  $^{14}\text{C}$  or  $^{125}\text{I}$ ) sterol (*e.g.*, cholesterol) or  $5\alpha$ -stanol and with a sample to be tested for the presence of an ligand; and (b) measuring the amount of detectably labeled sterol (*e.g.*, cholesterol) or  $5\alpha$ -stanol in the cell; wherein an NPC1L1 antagonist in the sample is identified by measuring substantially reduced detectably labeled sterol (*e.g.*, cholesterol) or  $5\alpha$ -stanol within the host cell, compared to what would be measured in the absence of such an antagonist and wherein an NPC1L1 agonist in the sample is identified by measuring substantially increased detectably labeled sterol (*e.g.*, cholesterol) or  $5\alpha$ -stanol within the host cell, compared to what would be measured in the absence of such an agonist.

The present invention includes methods for inhibiting NPC1L1-mediated intestinal sterol (*e.g.*, cholesterol) or  $5\alpha$ -stanol uptake, in a subject, by administering a substance identified by the screening methods described herein to the subject. Such substances include compounds such as small molecule antagonists of NPC1L1 other than ezetimibe. Also contemplated are methods for antagonizing NPC1L1-mediated sterol (*e.g.*, cholesterol) or  $5\alpha$ -stanol absorption by administering anti-NPC1L1 antibodies. NPC1L1-mediated absorption of sterol (*e.g.*, cholesterol) or

5α-stanol can also be antagonized by any method which reduces expression of NPC1L1 in an organism. For example, NPC1L1 expression can be reduced by introduction of anti-sense *NPC1L1* mRNA into a cell of an organism or by genetic mutation of the *NPC1L1* gene in an organism (*e.g.*, by complete knockout, disruption, truncation or by introduction of one or more point mutations).

Also included in the present invention is a mutant transgenic mammal (*e.g.*, mouse, rat, dog, rabbit, pig, guinea pig, cat, horse), preferably a mouse comprising a homozygous or heterozygous mutation (*e.g.*, disruption, truncation, one or more point mutations, knock out) of endogenous, chromosomal *NPC1L1* wherein, preferably, the mouse does not produce any functional NPC1L1 protein. Preferably, the mutant mouse, lacking functional NPC1L1, exhibits a reduced level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and/or a reduced level of serum sterol (*e.g.*, cholesterol) or 5α-stanol and/or a reduced level of liver sterol (*e.g.*, cholesterol) or 5α-stanol as compared to that of a non-mutant mouse comprising functional NPC1L1. Preferably, in the mutant mouse chromosome, the region of *NPC1L1* (SEQ ID NO: 45) deleted is from nucleotide 790 to nucleotide 998. In one embodiment, *NPC1L1* (SEQ ID NO: 11) is deleted from nucleotide 767 to nucleotide 975. Any offspring or progeny of a parent *NPC1L1* mutant mouse (*i.e.*, *npc1l1*) of the invention which has inherited an *npc1l1* mutant allele is also part of the present invention.

The scope of the present invention also includes a method for screening a sample for an intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption antagonist comprising (a) feeding a sterol (*e.g.*, cholesterol) or 5α-stanol-containing substance (*e.g.*, comprising radiolabeled cholesterol, such as <sup>14</sup>C-cholesterol or <sup>3</sup>H-cholesterol) to a first and second mouse comprising a functional *NPC1L1* gene and to a third, mutant mouse lacking a functional *NPC1L1*; (b) administering the sample to the first mouse comprising a functional *NPC1L1* but not to the second mouse; (c) measuring the amount of sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the intestine of said first, second and third mouse (*e.g.*, by measuring serum cholesterol); and (d) comparing the levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in each mouse; wherein the sample is determined to contain the intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption antagonist when the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the first mouse and third mouse are

less than the amount of intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption in the second mouse.

The present invention also encompasses a kit comprising (a) a substituted azetidinone (*e.g.*, ezetimibe) in a pharmaceutical dosage form (*e.g.*, a pill or tablet comprising 10 mg substituted azetidinone (*e.g.*, ezetimibe)); and (b) information, for example in the form of an insert, indicating that NPC1L1 is a target of ezetimibe. The kit may also include simvastatin in a pharmaceutical dosage form (*e.g.*, a pill or tablet comprising 5 mg, 10 mg, 20 mg, 40 mg or 80 mg simvastatin). The simvastatin in pharmaceutical dosage form and the ezetimibe in pharmaceutical dosage form can be associated in a single pill or tablet or in separate pills or tablets.

The present invention also provides any isolated mammalian cell (*e.g.*, isolated mouse cell, isolated rat cell or isolated human cell) which lacks a gene which encodes or can produce a functional NPC1L1 polypeptide. The isolated cell can be isolated from a mutant mouse comprising a homozygous mutation of endogenous, chromosomal *NPC1L1* wherein the mouse does not produce any functional NPC1L1 protein. Further, the mutation can be in a gene which when un-mutated encodes an amino acid sequence of SEQ ID NO: 12 (*e.g.*, comprising a nucleotide sequence of SEQ ID NO: 11). The cell can be isolated or derived from duodenum, gall bladder, liver, small intestine or stomach tissue. The cell can be an enterocyte.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows an equilibrium saturation binding plot exhibiting the binding of <sup>3</sup>H-EZE-glucuronide to rhesus brush border membrane (BBM) vesicles. Observed total binding (Total) is shown as open circles; nonspecific binding (NS) as triangles, and specific binding (S-bind) as solid circles.

Figure 1B shows a scatchard analysis of <sup>3</sup>H-EZE-glucuronide binding to rhesus brush border membrane vesicles.

Figure 2A shows an equilibrium saturation binding plot exhibiting the binding of <sup>3</sup>H-EZE-glucuronide (1) to rat brush border membrane vesicles. Observed total binding (open circles) and nonspecific binding (triangles), determined in the presence of 100  $\mu$ M unlabeled ezetimibe glucuronide, are included; specific binding (solid circles) was assessed from the difference between total and nonspecific binding.



Binding was measured at 2.5 mg protein/ml in a volume of 100  $\mu$ l after 1 hour incubation. Data were fit by nonlinear regression as described in Methods.

Figure 2B shows scatchard analysis of  $^3\text{H}$ -EZE-glucuronide binding to rat brush border membrane vesicles. The binding data identify a single high-affinity site with  $K_D = 542$  nM and  $B_{\text{max}} = 20.7$  pmol/mg protein.

Figure 3A shows association kinetic analysis of  $^3\text{H}$ -EZE-glucuronide in rat brush border membrane vesicles. Conditions were 25 nM of 1 and 3 mg/ml protein at 25°C. The second-order rate constant  $k_{\text{on}}$  ( $0.55 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ ) was calculated from  $k_{\text{obs}}$  ( $0.004 \text{ s}^{-1}$ ) as described in Methods.

Figure 3B shows dissociation kinetic analysis of  $^3\text{H}$ -EZE-glucuronide 1 in rat brush border membrane vesicles. After the complex was formed by incubating 25 nM of 1 and 3 mg/ml protein for 1 hour, dissociation was initiated by competition with 100  $\mu$ M unlabeled ezetimibe glucuronide. The curve is theoretical for  $k_{\text{off}} = 0.0024 \text{ s}^{-1}$ .

Figure 4A shows association kinetic analysis of  $^3\text{H}$ -EZE-glucuronide in rhesus brush border membrane vesicles.

Figure 4B shows dissociation kinetic analysis of  $^3\text{H}$ -EZE-glucuronide in rhesus brush border membrane vesicles.

Figure 5 shows the results of a binding assay where  $^3\text{H}$ -EZE-glucuronide is dissociated by EZE-glucuronide and compound 2 from rhesus (A) and rat (B) brush border membrane vesicles.

Figure 6 shows the results of a binding assay where  $^{35}\text{S}$ -2 is dissociated by EZE-glucuronide and 2 from mouse brush border membrane vesicles.

Figure 7 shows the distribution of  $^3\text{H}$ -EZE-glucuronide binding to rhesus (A) and rat (B) brush border membranes prepared from various portions of rhesus (A) and rat (B) intestinal tissue.

Figure 8 shows the results of a binding assay where  $^{35}\text{S}$ -2 is dissociated by EZE-glucuronide and various analogs from CHO cells transfected with rat NPC1L1.

Figure 9 shows the results of a binding assay where  $^{35}\text{S}$ -2 is dissociated by EZE-glucuronide and various analogs from CHO cells transfected with human NPC1L1.

Figure 10 shows the binding of  $^{35}\text{S}$ -2 to brush border membrane vesicles prepared from wild type (A) and *NPC1L1* knockout (-/-) mice.

Figure 11 shows the results of a binding assay where  $^{35}\text{S}$ -2 is dissociated by compound 2 from mouse wild type and *NPC1L1* knockout (-/-) brush  
5 border membrane vesicles.

Figure 12A shows equilibrium determination of  $K_D$  for ezetimibe glucuronide by competition of unlabeled compound against 1 in rat enterocyte brush border membranes. Membranes (1.5 mg/ml protein) were incubated with 1 (50 nM) and the indicated concentrations of ezetimibe glucuronide for 1 hour to ensure  
10 equilibrium.  $K_D$  at equilibrium is 600 nM. Figure 12B shows the corresponding measurement for rhesus monkey, which were conducted between 0.5 and 1.25 mg/ml protein and 22-50 nM 1, with incubation time of more than 3 hours.  $K_D$  at equilibrium is 38.6 nM.

Figure 13 shows the expression of NPC1L1 in HEK-293 cells using  
15 Western blot analysis (Panel 1) and immunofluorescence (Panel 2).

Figure 14A shows binding of  $^3\text{H}$ -ezetimibe glucuronide to enterocyte brush border membranes from wild type mice and NPC1L1 deficient mice in the presence of detergent. Figure 14B shows competition studies of unlabeled ezetimibe glucuronide against labeled ezetimibe glucuronide.

Figure 15 shows the effect of detergents, taurocholate and digitonin, on  
20 [ $^3\text{H}$ ]ezetimibe glucuronide binding.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention includes NPC1L1 polypeptides from rat, human and mouse, along with polynucleotides encoding the respective polypeptides.  
25 Preferably, the rat NPC1L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 2, the human NPC1L1 comprises the amino acid sequence set forth in SEQ ID NO: 4 and the mouse NPC1L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 12. The rat *NPC1L1* polynucleotide of SEQ ID NO: 1 or 10 encodes the rat NPC1L1 polypeptide. The human *NPC1L1*  
30 polynucleotide of SEQ ID NO: 3 encodes the human NPC1L1 polypeptide. The mouse *NPC1L1* polynucleotide of SEQ ID NO: 11 or 13 encodes the mouse NPC1L1 polypeptide.

The present invention includes any isolated polynucleotide or isolated polypeptide comprising a nucleotide or amino acid sequence referred to, below, in Table 1.

**Table 1. Polynucleotides and Polypeptides of the Invention.**

Polynucleotide or Polypeptide	Sequence Identifier
Rat <i>NPC1L1</i> polynucleotide	SEQ ID NO: 1
Rat <i>NPC1L1</i> polypeptide	SEQ ID NO: 2
Human <i>NPC1L1</i> polynucleotide	SEQ ID NO: 3
Human <i>NPC1L1</i> polypeptide	SEQ ID NO: 4
Rat <i>NPC1L1</i> expressed sequence tag 603662080F1 (partial sequence)	SEQ ID NO: 5
Rat <i>NPC1L1</i> expressed sequence tag 603665037F1 (partial sequence)	SEQ ID NO: 6
Rat <i>NPC1L1</i> expressed sequence tag 604034587F1 (partial sequence)	SEQ ID NO: 7
EST 603662080F1 with downstream sequences added	SEQ ID NO: 8
EST 603662080F1 with upstream and downstream sequences added	SEQ ID NO: 9
Back-translated polynucleotide sequence of rat <i>NPC1L1</i>	SEQ ID NO: 10
Mouse <i>NPC1L1</i> polynucleotide	SEQ ID NO: 11
Mouse <i>NPC1L1</i> polypeptide	SEQ ID NO: 12
Back-translated polynucleotide sequence of mouse <i>NPC1L1</i>	SEQ ID NO: 13
Back-translated polynucleotide sequence of human <i>NPC1L1</i>	SEQ ID NO: 51

5

A human *NPC1L1* is also disclosed under Genbank Accession Number AF192522. As discussed below, the nucleotide sequence of the rat *NPC1L1* set forth in SEQ ID NO: 1 was obtained from an expressed sequence tag (EST) from a rat jejunum enterocyte cDNA library. SEQ ID NOs: 5-7 include partial nucleotide

sequences of three independent cDNA clones. The downstream sequence of the SEQ ID NO: 5 EST (603662080F1) were determined; the sequencing data from these experiments are set forth in SEQ ID NO: 8. The upstream sequences were also determined; these data are set forth in SEQ ID NO: 9.

5                   SEQ ID NOs: 43 and 44 are the nucleotide and amino acid sequence, respectively, of human NPC1L1 which is disclosed under Genbank Accession No.: AF192522 (see Davies, *et al.*, (2000) Genomics 65(2): 137-45).

                  SEQ ID NO: 45 is the nucleotide sequence of a mouse *NPC1L1* which is disclosed under Genbank Accession No. AK078947.

10                  NPC1L1 mediates intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption. Inhibition of NPC1L1 in a patient is a useful method for reducing intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption and serum sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol in the patient. Reducing the level of intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption and serum sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol  
15 in a patient is a useful way in which to treat or prevent the occurrence of atherosclerosis, particularly diet-induced atherosclerosis.

                  As used herein, the term "sterol" includes, but is not limited to, cholesterol and phytosterols (including, but not limited to, sitosterol, campesterol, stigmasterol and avenosterol).

20                  As used herein, the term "5 $\alpha$ -stanol" includes, but is not limited to, cholestanol, 5 $\alpha$ -campestanol and 5 $\alpha$ -sitostanol.

                  Without being limited by the present hypothesis, the examples present a better understanding of the putative molecular interaction between NPC1L1 and cholesterol. In this regard, one of the more interesting features of NPC1L1 is that it  
25 contains the sterol-sensing domain (SSD) originally observed in SCAP (SREBP cleavage-activating protein). SCAP controls activation of sterol regulatory element binding proteins (SREBP), a transcription factor which controls more than 35 genes related to lipid and cholesterol homeostasis (Brown, M.S. & Goldstein, J.L. A proteolytic pathway that controls the cholesterol content of membranes, cells, and  
30 blood. *Proc. Natl. Acad. Sci. U.S.A.* 96, 11041-11048 (1999)). The SSD, consisting of ~180 amino acids in a packet of 5 putative membrane-spanning helices, also serves a regulatory function in two key enzymes on the cholesterol biosynthesis pathway and is present in the receptor Patched. Recently, high affinity binding of cholesterol to the

- SSD on SCAP has been demonstrated (Radhakrishnan, A., Sun, L., Kwon, H.J., Brown, M.S. & Goldstein, J.L., "Direct binding of cholesterol to the purified membrane region of SCAP: Mechanism for a sterol-sensing domain," *Mol. Cell* 15, 259-268 (2004)), suggesting that cholesterol may similarly bind to the SSD of
- 5 NPC1L1, and raising the possibility that ezetimibe may compete with cholesterol for binding at this site.

### Molecular Biology

- In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques
- 10 within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook, *et al.*, 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait, ed.
- 15 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins, eds. (1985)); Transcription And Translation (B.D. Hames & S.J. Higgins, eds. (1984)); Animal Cell Culture (R.I. Freshney, ed. (1986)); Immobilized Cells And Enzymes (IRL Press, (1986)); B. Perbal, A Practical Guide To Molecular Cloning (1984); F.M. Ausubel, *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).
- 20 The back-translated sequences of SEQ ID NO: 10 and of SEQ ID NO: 13 uses the single-letter code shown in Table 1 of Annex C, Appendix 2 of the PCT Administrative Instruction in the Manual of Patent Examination Procedure.

- A "polynucleotide", "nucleic acid" or "nucleic acid molecule" may refer to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine,
- 25 uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in single stranded form, double-stranded form or otherwise.

- A "polynucleotide sequence", "nucleic acid sequence" or "nucleotide
- 30 sequence" is a series of nucleotide bases (also called "nucleotides") in a nucleic acid, such as DNA or RNA, and means any chain of two or more nucleotides.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in production of the product.

5 The term "gene" means a DNA sequence that codes for or corresponds to a particular sequence of ribonucleotides or amino acids which comprise all or part of one or more RNA molecules, proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine, for example, the conditions under which the gene is expressed. Genes may be transcribed from DNA to RNA which may or may not be translated into an amino acid sequence.

10 The present invention includes nucleic acid fragments of any of SEQ ID NOs: 1, 5-11 or 13. A nucleic acid "fragment" includes at least about 30 (*e.g.*, 31, 32, 33, 34), preferably at least about 35 (*e.g.*, 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34), more preferably at least about 45 (*e.g.*, 35, 36, 37, 38, 39, 40, 41, 42, 43 or 44), and most preferably at least about 126 or more contiguous nucleotides (*e.g.*, 130, 131,  
15 132, 133, 134, 135, 136, 137, 138, 139, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 1000 or 1200) from any of SEQ ID NOs: 1, 5-11 or 13.

The present invention also includes nucleic acid fragments consisting of at least about 7 (*e.g.*, 9, 12, 17, 19), preferably at least about 20 (*e.g.*, 30, 40, 50, 60), more preferably about 70 (*e.g.*, 80, 90, 95), yet more preferably at least about 100  
20 (*e.g.*, 105, 110, 114) and even more preferably at least about 115 (*e.g.*, 117, 119, 120, 122, 124, 125, 126) contiguous nucleotides from any of SEQ ID NOs: 1, 5-11 or 13.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of no more than about 100 nucleotides (*e.g.*, 30, 40, 50, 60, 70, 80, or 90), that may be hybridizable to a genomic DNA molecule, a cDNA molecule, or an  
25 mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides can be labeled, *e.g.*, by incorporation of <sup>32</sup>P-nucleotides, <sup>3</sup>H-nucleotides, <sup>14</sup>C-nucleotides, <sup>35</sup>S-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another  
30 embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of the gene, or to detect the presence of nucleic acids. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer.

A "protein sequence", "peptide sequence" or "polypeptide sequence" or "amino acid sequence" may refer to a series of two or more amino acids in a protein, peptide or polypeptide.

"Protein", "peptide" or "polypeptide" includes a contiguous string of  
5 two or more amino acids. Preferred peptides of the invention include those set forth in any of SEQ ID NOs: 2 or 12 as well as variants and fragments thereof. Such fragments preferably comprise at least about 10 (*e.g.*, 11, 12, 13, 14, 15, 16, 17, 18 or 19), more preferably at least about 20 (*e.g.*, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40), and yet more preferably at least about 42 (*e.g.*, 43, 44, 45, 46, 47, 48, 49, 50, 60,  
10 70, 80, 90, 100, 110, 120 or 130) or more contiguous amino acid residues from any of SEQ ID NOs: 2 or 12.

The present invention also includes polypeptides, preferably antigenic polypeptides, consisting of at least about 7 (*e.g.*, 9, 10, 13, 15, 17, 19), preferably at least about 20 (*e.g.*, 22, 24, 26, 28), yet more preferably at least about 30 (*e.g.*, 32, 34,  
15 36, 38) and even more preferably at least about 40 (*e.g.*, 41, 42) contiguous amino acids from any of SEQ ID NOs: 2 or 12.

The polypeptides of the invention can be produced by proteolytic cleavage of an intact peptide, by chemical synthesis or by the application of recombinant DNA technology and are not limited to polypeptides delineated by  
20 proteolytic cleavage sites. The polypeptides, either alone or cross-linked or conjugated to a carrier molecule to render them more immunogenic, are useful as antigens to elicit the production of antibodies and fragments thereof. The antibodies can be used, *e.g.*, in immunoassays for immunoaffinity purification or for inhibition of NPC1L1, etc.

25 The terms "isolated polynucleotide" or "isolated polypeptide" include a polynucleotide (*e.g.*, RNA or DNA molecule, or a mixed polymer) or a polypeptide, respectively, which are partially or fully separated from other components that are normally found in cells or in recombinant DNA expression systems. These components include, but are not limited to, cell membranes, cell walls, ribosomes,  
30 polymerases, serum components and extraneous genomic sequences.

An isolated polynucleotide or polypeptide will, preferably, be an essentially homogeneous composition of molecules but may contain some heterogeneity.

"Amplification" of DNA as used herein may denote the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. For a description of PCR see Saiki, *et al.*, Science (1988) 239: 487.

5                   The term "host cell" includes any cell of any organism that is selected, modified, transfected, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example, the expression or replication, by the cell, of a gene, a DNA or RNA sequence or a protein. Preferred host cells include HEK-293 cells, chinese hamster ovary (CHO) cells, murine macrophage J774 cells or  
10 any other macrophage cell line and human intestinal epithelial Caco2 cells.

                  The nucleotide sequence of a nucleic acid may be determined by any method known in the art (e.g., chemical sequencing or enzymatic sequencing). "Chemical sequencing" of DNA includes methods such as that of Maxam and Gilbert (1977) (Proc. Natl. Acad. Sci. USA 74: 560), in which DNA is randomly cleaved  
15 using individual base-specific reactions. "Enzymatic sequencing" of DNA includes methods such as that of Sanger (Sanger, *et al.*, (1977) Proc. Natl. Acad. Sci. USA 74: 5463).

                  The nucleic acids herein may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences,  
20 including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'- non-coding regions, and the like.

                  In general, a "promoter" or "promoter sequence" is a DNA regulatory region capable of binding an RNA polymerase in a cell (e.g., directly or through other  
25 promoter-bound proteins or substances) and initiating transcription of a coding sequence. A promoter sequence is, in general, bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at any level. Within the promoter sequence may be found a transcription initiation site  
30 (conveniently defined, for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. The promoter may be operably associated with other expression control sequences, including enhancer and repressor sequences or with a nucleic acid of the



invention. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and 5,168,062), the SV40 early promoter region (Benoist, *et al.*, (1981) *Nature* 290: 304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, (1980) *Cell* 22: 787-797), the herpes thymidine kinase promoter (Wagner, *et al.*, (1981) *Proc. Natl. Acad. Sci. USA* 78: 1441-1445), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, (1982) *Nature* 296: 39-42); prokaryotic expression vectors such as the  $\beta$ -lactamase promoter (Villa-Komaroff, *et al.*, (1978) *Proc. Natl. Acad. Sci. USA* 75: 3727-3731), or the *tac* promoter (DeBoer, *et al.*, (1983) *Proc. Natl. Acad. Sci. USA* 80: 21-25); see also "Useful proteins from recombinant bacteria" in *Scientific American* (1980) 242: 74-94; and promoter elements from yeast or other fungi such as the *Gal 4* promoter, the *ADC* (alcohol dehydrogenase) promoter, *PGK* (phosphoglycerol kinase) promoter or the alkaline phosphatase promoter.

15 A coding sequence is "under the control of", "functionally associated with" or "operably associated with" transcriptional and translational control sequences in a cell when the sequences direct RNA polymerase mediated transcription of the coding sequence into RNA, preferably mRNA, which then may be RNA spliced (if it contains introns) and, optionally, translated into a protein encoded by the coding sequence.

The terms "express" and "expression" mean allowing or causing the information in a gene, RNA or DNA sequence to become manifest; for example, producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene. A DNA sequence is expressed in or by a cell to form an "expression product" such as an RNA (*e.g.*, mRNA) or a protein. The expression product itself may also be said to be "expressed" by the cell.

The term "transformation" means the introduction of a nucleic acid into a cell. The introduced gene or sequence may be called a "clone". A host cell that receives the introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone." The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or from cells of a different genus or species.

The term "vector" includes a vehicle (e.g., a plasmid) by which a DNA or RNA sequence can be introduced into a host cell, so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence.

Vectors that can be used in this invention include plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles that may facilitate introduction of the nucleic acids into the genome of the host. Plasmids are the most commonly used form of vector but all other forms of vectors which serve a similar function and which are, or become, known in the art are suitable for use herein. See, e.g., Pouwels, *et al.*, Cloning Vectors: A Laboratory Manual, 1985 and Supplements, Elsevier, N.Y., and Rodriguez *et al.* (eds.), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, 1988, Butterworth, Boston, MA.

The term "expression system" means a host cell and compatible vector which, under suitable conditions, can express a protein or nucleic acid which is carried by the vector and introduced to the host cell. Common expression systems include *E. coli* host cells and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors.

Expression of nucleic acids encoding the NPC1L1 polypeptides of this invention can be carried out by conventional methods in either prokaryotic or eukaryotic cells. Although *E. coli* host cells are employed most frequently in prokaryotic systems, many other bacteria, such as various strains of *Pseudomonas* and *Bacillus*, are known in the art and can be used as well. Suitable host cells for expressing nucleic acids encoding the NPC1L1 polypeptides include prokaryotes and higher eukaryotes. Prokaryotes include both gram-negative and gram-positive organisms, e.g., *E. coli* and *B. subtilis*. Higher eukaryotes include established tissue culture cell lines from animal cells, both of non-mammalian origin, e.g., insect cells, and birds, and of mammalian origin, e.g., human, primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. A representative vector for amplifying DNA is pBR322 or many of its derivatives (e.g., pUC18 or 19). Vectors that can be used to express the NPC1L1 polypeptides include, but are not limited to, those containing the *lac* promoter (pUC-series); *trp* promoter (pBR322-*trp*); *Ipp* promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as *ptac* (pDR540). See Brosius *et al.*, "Expression Vectors Employing Lambda-, *trp*-, *lac*-, and *Ipp*-derived

Promoters", in Rodriguez and Denhardt (eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses. 1988, Butterworth, Boston, pp. 205-236. Many polypeptides can be expressed, at high levels, in an *E.coli*/T7 expression system as disclosed in U.S. Patent Nos. 4,952,496; 5,693,489 and 5,869,320 and in Davanloo, P., *et al.*, (1984) Proc. Natl. Acad. Sci. USA 81: 2035-2039; Studier, F.W., *et al.*, (1986) J. Mol. Biol. 189: 113-130; Rosenberg, A. H., *et al.*, (1987) Gene 56: 125-135; and Dunn, J.J., *et al.*, (1988) Gene 68: 259.

Higher eukaryotic tissue culture cells may also be used for the recombinant production of the NPC1L1 polypeptides of the invention. Although any higher eukaryotic tissue culture cell line might be used, including insect baculovirus expression systems, mammalian cells are preferred. Transformation or transfection and propagation of such cells have become a routine procedure. Examples of useful cell lines include HeLa cells, chinese hamster ovary (CHO) cell lines, J774 cells, HEK-293 cells, Caco2 cells, baby rat kidney (BRK) cell lines, insect cell lines, bird cell lines, and monkey (COS) cell lines. Expression vectors for such cell lines usually include an origin of replication, a promoter, a translation initiation site, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a transcription termination site. These vectors also, usually, contain a selection gene or amplification gene. Suitable expression vectors may be plasmids, viruses, or retroviruses carrying promoters derived, *e.g.*, from such sources as adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus. Examples of expression vectors include pCR@3.1, pCDNA1, pCD (Okayama, *et al.*, (1985) Mol. Cell Biol. 5: 1136), pMC1neo Poly-A (Thomas, *et al.*, (1987) Cell 51: 503), pREP8, pSVSPORT and derivatives thereof, and baculovirus vectors such as pAC373 or pAC610. One embodiment of the invention includes membrane bound NPC1L1. In this embodiment, NPC1L1 can be expressed in the cell membrane of a eukaryotic cell and the membrane bound protein can be isolated from the cell by conventional methods which are known in the art.

The present invention also includes fusions which include the NPC1L1 polypeptides and *NPC1L1* polynucleotides of the present invention and a second polypeptide or polynucleotide moiety, which may be referred to as a "tag". The fusions of the present invention may comprise any of the polynucleotides or polypeptides set forth in Table 1 or any subsequence or fragment thereof (discussed

above). The fused polypeptides of the invention may be conveniently constructed, for example, by insertion of a polynucleotide of the invention or fragment thereof into an expression vector. The fusions of the invention may include tags which facilitate purification or detection. Such tags include glutathione-S-transferase (GST),  
5 hexahistidine (His6) tags, maltose binding protein (MBP) tags, haemagglutinin (HA) tags, cellulose binding protein (CBP) tags and myc tags. Detectable tags such as  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^3\text{H}$ ,  $^{99\text{m}}\text{Tc}$ ,  $^{123}\text{I}$ ,  $^{111}\text{In}$ ,  $^{68}\text{Ga}$ ,  $^{18}\text{F}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{113\text{m}}\text{In}$ ,  $^{76}\text{Br}$ ,  $^{67}\text{Ga}$ ,  $^{99\text{m}}\text{Tc}$ ,  $^{123}\text{I}$ ,  $^{111}\text{In}$  and  $^{68}\text{Ga}$  may also be used to label the polypeptides and polynucleotides of the invention. Methods for constructing and using such fusions are very conventional and well  
10 known in the art.

Modifications (e.g., post-translational modifications) that occur in a polypeptide often will be a function of how it is made. For polypeptides made by expressing a cloned gene in a host, for instance, the nature and extent of the modifications, in large part, will be determined by the host cell's post-translational  
15 modification capacity and the modification signals present in the polypeptide amino acid sequence. For instance, as is well known, glycosylation often does not occur in bacterial hosts such as *E. coli*. Accordingly, when glycosylation is desired, a polypeptide can be expressed in a glycosylating host, generally a eukaryotic cell. Insect cells often carry out post-translational glycosylations which are similar to those  
20 of mammalian cells. For this reason, insect cell expression systems have been developed to express, efficiently, mammalian proteins having native patterns of glycosylation. An insect cell which may be used in this invention is any cell derived from an organism of the class *Insecta*. Preferably, the insect is *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusia ni* (High 5). Examples of insect expression  
25 systems that can be used with the present invention, for example to produce NPC1L1 polypeptide, include Bac-To-Bac (Invitrogen Corporation, Carlsbad, CA) or Gateway (Invitrogen Corporation, Carlsbad, CA). If desired, deglycosylation enzymes can be used to remove carbohydrates attached during production in eukaryotic expression systems.

30 Other modifications may also include addition of aliphatic esters or amides to the polypeptide carboxyl terminus. The present invention also includes analogs of the NPC1L1 polypeptides which contain modifications, such as incorporation of unnatural amino acid residues, or phosphorylated amino acid

residues such as phosphotyrosine, phosphoserine or phosphothreonine residues. Other potential modifications include sulfonation, biotinylation, or the addition of other moieties. For example, the NPC1L1 polypeptides of the invention may be appended with a polymer which increases the half-life of the peptide in the body of a  
5 subject. Preferred polymers include polyethylene glycol (PEG) (*e.g.*, PEG with a molecular weight of 2 kDa, 5 kDa, 10 kDa, 12 kDa, 20 kDa, 30 kDa and 40 kDa), dextran and monomethoxypolyethylene glycol (mPEG).

The peptides of the invention may also be cyclized. Specifically, the amino- and carboxy-terminal residues of an NPC1L1 polypeptide or two internal  
10 residues of an NPC1L1 polypeptide of the invention can be fused to create a cyclized peptide. Methods for cyclizing peptides are conventional and very well known in the art; for example, see Gurrath, *et al.*, (1992) Eur. J. Biochem. 210: 911-921.

The present invention contemplates any superficial or slight modification to the amino acid or nucleotide sequences which correspond to the  
15 polypeptides of the invention. In particular, the present invention contemplates sequence conservative variants of the nucleic acids which encode the polypeptides of the invention. "Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon results in no alteration in the amino acid encoded at that position. Function-conservative variants  
20 of the polypeptides of the invention are also contemplated by the present invention. "Function-conservative variants" are those in which one or more amino acid residues in a protein or enzyme have been changed without altering the overall conformation and function of the polypeptide, including, but, by no means, limited to, replacement of an amino acid with one having similar properties. Amino acids with similar  
25 properties are well known in the art. For example, polar/hydrophilic amino acids which may be interchangeable include asparagine, glutamine, serine, cysteine, threonine, lysine, arginine, histidine, aspartic acid and glutamic acid; nonpolar/hydrophobic amino acids which may be interchangeable include glycine, alanine, valine, leucine, isoleucine, proline, tyrosine, phenylalanine, tryptophan and  
30 methionine; acidic amino acids, which may be interchangeable include aspartic acid and glutamic acid and basic amino acids, which may be interchangeable include histidine, lysine and arginine.

The present invention includes polynucleotides encoding rat, human or mouse NPC1L1 and fragments thereof as well as nucleic acids which hybridize to the polynucleotides. Preferably, the nucleic acids hybridize under low stringency conditions, more preferably under moderate stringency conditions and most preferably under high stringency conditions. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook, *et al.*, supra). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Typical low stringency hybridization conditions are 55°C, 5X SSC, 0.1% SDS, 0.25% milk, and no formamide at 42°C; or 30% formamide, 5X SSC, 0.5% SDS at 42°C. Typical, moderate stringency hybridization conditions are similar to the low stringency conditions except the hybridization is carried out in 40% formamide, with 5X or 6X SSC at 42°C. High stringency hybridization conditions are similar to low stringency conditions except the hybridization conditions are carried out in 50% formamide, 5X or 6X SSC and, optionally, at a higher temperature (*e.g.*, higher than 42°C: 57°C, 59°C, 60°C, 62°C, 63°C, 65°C or 68°C). In general, SSC is 0.15M NaCl and 0.015M Na-citrate. Hybridization requires that the two nucleic acids contain complementary sequences, although, depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the higher the stringency under which the nucleic acids may hybridize. For hybrids of greater than 100 nucleotides in length, equations for calculating the melting temperature have been derived (see Sambrook, *et al.*, supra, 9.50-9.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook, *et al.*, supra).

Also included in the present invention are polynucleotides comprising nucleotide sequences and polypeptides comprising amino acid sequences which are at least about 70% identical, preferably at least about 80% identical, more preferably at least about 90% identical and most preferably at least about 95% identical (*e.g.*, 95%,

96%, 97%, 98%, 99%, 100%) to the reference rat *NPC1L1* nucleotide (*e.g.*, any of SEQ ID NOs: 1 or 5-10) and amino acid sequences (*e.g.*, SEQ ID NO: 2), reference human *NPC1L1* nucleotide (*e.g.*, SEQ ID NO: 3) and amino acid sequences (*e.g.*, SEQ ID NO: 4) or the reference mouse *NPC1L1* nucleotide (*e.g.*, any of SEQ ID NOs: 11 or 13) and amino acid sequences (*e.g.*, SEQ ID NO: 12), when the comparison is performed by a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences. Polypeptides comprising amino acid sequences which are at least about 70% similar, preferably at least about 80% similar, more preferably at least about 90% similar and most preferably at least about 95% similar (*e.g.*, 95%, 96%, 97%, 98%, 99%, 100%) to the reference rat NPC1L1 amino acid sequence of SEQ ID NO: 2, reference human NPC1L1 amino acid sequence of SEQ ID NO: 4 or the reference mouse NPC1L1 amino acid sequence of SEQ ID NO: 12, when the comparison is performed with a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences, are also included in the present invention.

Sequence identity refers to exact matches between the nucleotides or amino acids of two sequences which are being compared. Sequence similarity refers to both exact matches between the amino acids of two polypeptides which are being compared in addition to matches between nonidentical, biochemically related amino acids. Biochemically related amino acids which share similar properties and may be interchangeable are discussed above.

The following references regarding the BLAST algorithm are herein incorporated by reference: **BLAST ALGORITHMS**: Altschul, S.F., *et al.*, (1990) J. Mol. Biol. 215: 403-410; Gish, W., *et al.*, (1993) Nature Genet. 3: 266-272; Madden, T.L., *et al.*, (1996) Meth. Enzymol. 266: 131-141; Altschul, S.F., *et al.*, (1997) Nucleic Acids Res. 25: 3389-3402; Zhang, J., *et al.*, (1997) Genome Res. 7: 649-656; Wootton, J.C., *et al.*, (1993) Comput. Chem. 17: 149-163; Hancock, J.M., *et al.*, (1994) Comput. Appl. Biosci. 10: 67-70; **ALIGNMENT SCORING SYSTEMS**: Dayhoff, M.O., *et al.*, "A model of evolutionary change in proteins" in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 345-352, Natl. Biomed. Res. Found., Washington, DC; Schwartz, R.M., *et al.*, "Matrices

for detecting distant relationships" in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 353-358, Natl. Biomed. Res. Found., Washington, DC; Altschul, S.F., (1991) J. Mol. Biol. 219: 555-565; States, D.J., *et al.*, (1991) Methods 3: 66-70; Henikoff, S., *et al.*, (1992) Proc. Natl. Acad. Sci. USA 89: 10915-10919; Altschul, S.F., *et al.*, (1993) J. Mol. Evol. 36: 290-300; **ALIGNMENT STATISTICS**: Karlin, S., *et al.*, (1990) Proc. Natl. Acad. Sci. USA 87: 2264-2268; Karlin, S., *et al.*, (1993) Proc. Natl. Acad. Sci. USA 90: 5873-5877; Dembo, A., *et al.*, (1994) Ann. Prob. 22: 2022-2039; and Altschul, S.F. "Evaluating the statistical significance of multiple distinct local alignments" in Theoretical and Computational Methods in Genome Research (S. Suhai, ed.), (1997) pp. 1-14, Plenum, New York.

### **Protein Purification**

The proteins, polypeptides and antigenic fragments of this invention can be purified by standard methods, including, but not limited to, salt or alcohol precipitation, affinity chromatography (*e.g.*, used in conjunction with a purification tagged NPC1L1 polypeptide as discussed above), preparative disc-gel electrophoresis, isoelectric focusing, high pressure liquid chromatography (HPLC), reversed-phase HPLC, gel filtration, cation and anion exchange and partition chromatography, and countercurrent distribution. Such purification methods are well known in the art and are disclosed, *e.g.*, in "Guide to Protein Purification", Methods in Enzymology, Vol. 182, M. Deutscher, Ed., 1990, Academic Press, New York, NY.

Purification steps can be followed by performance of assays for receptor binding activity as described below. Particularly where an NPC1L1 polypeptide is being isolated from a cellular or tissue source, it is preferable to include one or more inhibitors of proteolytic enzymes in the assay system, such as phenylmethanesulfonyl fluoride (PMSF), Pefabloc SC, pepstatin, leupeptin, chymostatin and EDTA.

### **Antibody Molecules**

Antigenic (including immunogenic) fragments of the NPC1L1 polypeptides of the invention are within the scope of the present invention (*e.g.*, 42 or more contiguous amino acids from SEQ ID NO: 2, 4 or 12). The antigenic peptides may be useful, *inter alia*, for preparing isolated antibody molecules which recognize NPC1L1. Isolated anti-NPC1L1 antibody molecules are useful NPC1L1 ligands.



An antigen is any molecule that can bind specifically to an antibody. Some antigens cannot, by themselves, elicit antibody production. Those that can induce antibody production are immunogens.

Preferably, isolated anti-NPC1L1 antibodies recognize an antigenic peptide comprising an amino acid sequence selected from SEQ ID NOs: 39-42 (*e.g.*,  
5 an antigen derived from rat NPC1L1). More preferably, the antibody is A0715, A0716, A0717, A0718, A0867, A0868, A1801 or A1802.

The term "antibody molecule" includes, but is not limited to, antibodies and fragments (preferably antigen-binding fragments) thereof. The term  
10 includes monoclonal antibodies, polyclonal antibodies, bispecific antibodies, Fab antibody fragments, F(ab)<sub>2</sub> antibody fragments, Fv antibody fragments (*e.g.*, V<sub>H</sub> or V<sub>L</sub>), single chain Fv antibody fragments and dsFv antibody fragments. Furthermore, the antibody molecules of the invention may be fully human antibodies, mouse antibodies, rat antibodies, rabbit antibodies, goat antibodies, chicken antibodies,  
15 humanized antibodies or chimeric antibodies.

Although it is not always necessary, when NPC1L1 polypeptides are used as antigens to elicit antibody production in an immunologically competent host, smaller antigenic fragments are, preferably, first rendered more immunogenic by cross-linking or concatenation, or by coupling to an immunogenic carrier molecule  
20 (*i.e.*, a macromolecule having the property of independently eliciting an immunological response in a host animal, such as diphtheria toxin or tetanus). Cross-linking or conjugation to a carrier molecule may be required because small polypeptide fragments sometimes act as haptens (molecules which are capable of specifically binding to an antibody but incapable of eliciting antibody production, *i.e.*,  
25 they are not immunogenic). Conjugation of such fragments to an immunogenic carrier molecule renders them more immunogenic through what is commonly known as the "carrier effect".

Carrier molecules include, *e.g.*, proteins and natural or synthetic polymeric compounds such as polypeptides, polysaccharides, lipopolysaccharides,  
30 etc. Protein carrier molecules are especially preferred, including, but not limited to, keyhole limpet hemocyanin and mammalian serum proteins such as human or bovine gammaglobulin, human, bovine or rabbit serum albumin, or methylated or other derivatives of such proteins. Other protein carriers will be apparent to those skilled in

the art. Preferably, the protein carrier will be foreign to the host animal in which antibodies against the fragments are to be elicited.

Covalent coupling to the carrier molecule can be achieved using methods well known in the art, the exact choice of which will be dictated by the nature of the carrier molecule used. When the immunogenic carrier molecule is a protein, the fragments of the invention can be coupled, *e.g.*, using water-soluble carbodiimides such as dicyclohexylcarbodiimide or glutaraldehyde.

Coupling agents, such as these, can also be used to cross-link the fragments to themselves without the use of a separate carrier molecule. Such cross-linking into aggregates can also increase immunogenicity. Immunogenicity can also be increased by the use of known adjuvants, alone or in combination with coupling or aggregation.

Adjuvants for the vaccination of animals include, but are not limited to, Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate); Freund's complete or incomplete adjuvant; mineral gels such as aluminum hydroxide, aluminum phosphate and alum; surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl-N',N'-bis(2-hydroxymethyl) propanediamine, methoxyhexadecylglycerol and pluronic polyols; polyanions such as pyran, dextran sulfate, poly IC, polyacrylic acid and carbopol; peptides such as muramyl dipeptide, dimethylglycine and tuftsin; and oil emulsions. The polypeptides could also be administered following incorporation into liposomes or other microcarriers.

Information concerning adjuvants and various aspects of immunoassays are disclosed, *e.g.*, in the series by P. Tijssen, Practice and Theory of Enzyme Immunoassays, 3rd Edition, 1987, Elsevier, New York. Other useful references covering methods for preparing polyclonal antisera include Microbiology, 1969, Hoeber Medical Division, Harper and Row; Landsteiner, Specificity of Serological Reactions, 1962, Dover Publications, New York, and Williams, *et al.*, Methods in Immunology and Immunochemistry, Vol. 1, 1967, Academic Press, New York.

The anti-NPC1L1 antibody molecules of the invention preferably recognize human, mouse or rat NPC1L1; however, the present invention includes antibody molecules which recognize NPC1L1 from any species, preferably mammals

(e.g., cat, sheep or horse). The present invention also includes complexes comprising an NPC1L1 polypeptide of the invention and an anti-NPC1L1 antibody molecule. Such complexes can be made by simply contacting the antibody molecule with its cognate polypeptide.

5                Various methods may be used to make the antibody molecules of the invention. Human antibodies can be made, for example, by methods which are similar to those disclosed in U.S. Patent Nos. 5,625,126; 5,877,397; 6,255,458; 6,023,010 and 5,874,299.

                 Hybridoma cells which produce the monoclonal anti-NPC1L1  
10    antibodies may be produced by methods which are commonly known in the art. These methods include, but are not limited to, the hybridoma technique originally developed by Kohler, *et al.*, (1975) (Nature 256: 495-497), as well as the trioma technique (Hering, *et al.*, (1988) Biomed. Biochim. Acta. 47: 211-216 and Hagiwara, *et al.*, (1993) Hum. Antibod. Hybridomas 4: 15), the human B-cell hybridoma  
15    technique (Kozbor, *et al.*, (1983) Immunology Today 4: 72 and Cote, *et al.*, (1983) Proc. Natl. Acad. Sci. U.S.A. 80: 2026-2030), and the EBV-hybridoma technique (Cole, *et al.*, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1985). ELISA may be used to determine if hybridoma cells are expressing anti-NPC1L1 antibodies.

20                The anti-NPC1L1 antibody molecules of the present invention may also be produced recombinantly (e.g., in an *E.coli*/T7 expression system as discussed above). In this embodiment, nucleic acids encoding the antibody molecules of the invention (e.g., V<sub>H</sub> or V<sub>L</sub>) may be inserted into a pet-based plasmid and expressed in the *E.coli*/T7 system. There are several methods by which to produce recombinant  
25    antibodies which are known in the art. An example of a method for recombinant production of antibodies is disclosed in U.S. Patent No. 4,816,567. See also Skerra, A., *et al.*, (1988) Science 240: 1038-1041; Better, M., *et al.*, (1988) Science 240: 1041-1043 and Bird, R.E., *et al.*, (1988) Science 242: 423-426.

                 The term "monoclonal antibody," includes an antibody obtained from a  
30    population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible, naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Monoclonal antibodies are

advantageous in that they may be synthesized by a hybridoma culture, essentially uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. The monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method as described by Kohler, *et al.*, (1975) Nature 256: 495.

The term "polyclonal antibody" includes an antibody which was produced among or in the presence of one or more other, non-identical antibodies. In general, polyclonal antibodies are produced from a B-lymphocyte in the presence of several other B-lymphocytes which produced non-identical antibodies. Typically, polyclonal antibodies are obtained directly from an immunized animal (*e.g.*, a rabbit).

A "bispecific antibody" comprises two different antigen binding regions which bind to distinct antigens. Bispecific antibodies, as well as methods of making and using the antibodies, are conventional and very well known in the art.

Anti-idiotypic antibodies or anti-idiotypes are antibodies directed against the antigen-combining region or variable region (called the idiotype) of another antibody molecule. As disclosed by Jerne (Jerne, N. K., (1974) Ann. Immunol. (Paris) 125c: 373 and Jerne, N. K., *et al.*, (1982) EMBO 1: 234), immunization with an antibody molecule expressing a paratope (antigen-combining site) for a given antigen (*e.g.*, NPC1L1) will produce a group of anti-antibodies, some of which share, with the antigen, a complementary structure to the paratope. Immunization with a subpopulation of the anti-idiotypic antibodies will, in turn, produce a subpopulation of antibodies or immune cell subsets that are reactive to the initial antigen.

The term "fully human antibody" refers to an antibody which comprises human immunoglobulin sequences only. Similarly, "mouse antibody" refers to an antibody which comprises mouse immunoglobulin sequences only.

"Human/mouse chimeric antibody" refers to an antibody which comprises a mouse variable region ( $V_H$  and  $V_L$ ) fused to a human constant region.

"Humanized" anti-NPC1L1 antibodies are also within the scope of the present invention. Humanized forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, which contain minimal sequence derived from non-

human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region of the recipient are replaced by residues from a complementary determining region of a nonhuman species (donor antibody), such as mouse, rat or rabbit, having a desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are also replaced by corresponding non-human residues.

"Single-chain Fv" or "sFv" antibody fragments include the V<sub>H</sub> and/or V<sub>L</sub> domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the sFv polypeptide further comprises a polypeptide linker between the V<sub>H</sub> and V<sub>L</sub> domains which enables the sFv to form the desired structure for antigen binding. Techniques described for the production of single chain antibodies (U.S. Patent Nos. 5,476,786; 5,132,405 and 4,946,778) can be adapted to produce anti-NPC1L1 specific, single chain antibodies. For a review of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore, eds., Springer-Verlag, N.Y., pp. 269-315 (1994).

"Disulfide stabilized Fv fragments" and "dsFv" include molecules having a variable heavy chain (V<sub>H</sub>) and/or a variable light chain (V<sub>L</sub>) which are linked by a disulfide bridge.

Antibody fragments within the scope of the present invention also include F(ab)<sub>2</sub> fragments which may be produced by enzymatic cleavage of an IgG by, for example, pepsin. Fab fragments may be produced by, for example, reduction of F(ab)<sub>2</sub> with dithiothreitol or mercaptoethylamine.

An FV fragment is a V<sub>L</sub> or V<sub>H</sub> region.

Depending on the amino acid sequences of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are at least five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3 and IgG-4; IgA-1 and IgA-2.

The anti-NPC1L1 antibody molecules of the invention may also be conjugated to a chemical moiety. The chemical moiety may be, *inter alia*, a polymer, a radionuclide or a cytotoxic factor. Preferably, the chemical moiety is a polymer which increases the half-life of the antibody molecule in the body of a subject.

Suitable polymers include, but are by no means limited to, polyethylene glycol (PEG) (e.g., PEG with a molecular weight of 2kDa, 5kDa, 10kDa, 12kDa, 20kDa, 30kDa or 40kDa), dextran and monomethoxypolyethylene glycol (mPEG). Methods for producing PEGylated anti-IL8 antibodies which are described in U.S. Patent No.

- 5 6,133,426 can be applied to the production of PEGylated anti-NPC1L1 antibodies of the invention. Lee, *et al.*, (1999) (Bioconj. Chem. 10: 973-981) discloses PEG conjugated single-chain antibodies. Wen, *et al.*, (2001) (Bioconj. Chem. 12: 545-553) discloses conjugating antibodies with PEG which is attached to a radiometal chelator (diethylenetriaminopentaacetic acid (DTPA)).

- 10 The antibody molecules of the invention may also be conjugated with labels such as  $^{99}\text{Tc}$ ,  $^{90}\text{Y}$ ,  $^{111}\text{In}$ ,  $^{32}\text{P}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ ,  $^{131}\text{I}$ ,  $^{11}\text{C}$ ,  $^{15}\text{O}$ ,  $^{13}\text{N}$ ,  $^{18}\text{F}$ ,  $^{35}\text{S}$ ,  $^{51}\text{Cr}$ ,  $^{57}\text{Co}$ ,  $^{226}\text{Ra}$ ,  $^{60}\text{Co}$ ,  $^{59}\text{Fe}$ ,  $^{57}\text{Se}$ ,  $^{152}\text{Eu}$ ,  $^{67}\text{Cu}$ ,  $^{217}\text{Ci}$ ,  $^{211}\text{At}$ ,  $^{212}\text{Pb}$ ,  $^{47}\text{Sc}$ ,  $^{109}\text{Pd}$ ,  $^{234}\text{Th}$ ,  $^{40}\text{K}$ ,  $^{157}\text{Gd}$ ,  $^{55}\text{Mn}$ ,  $^{52}\text{Tr}$  or  $^{56}\text{Fe}$ .

- The antibody molecules of the invention may also be conjugated with  
15 fluorescent or chemiluminescent labels, including fluorophores such as rare earth chelates, fluorescein and its derivatives, rhodamine and its derivatives, isothiocyanate, phycoerythrin, phycocyanin, allophycocyanin, o-phthalaldehyde, fluorescamine,  $^{152}\text{Eu}$ , dansyl, umbelliferone, luciferin, luminal label, isoluminal label, an aromatic  
20 acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, an aequorin label, 2,3-dihydrophthalazinediones, biotin/avidin, spin labels and stable free radicals.

- The antibody molecules may also be conjugated to a cytotoxic factor such as diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A chain, ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins and  
25 compounds (e.g., fatty acids), dianthin proteins, *Phytolacca americana* proteins PAPI, PAPII, and PAP-S, *momordica charantia* inhibitor, curcin, croton, *saponaria officinalis* inhibitor, mitogellin, restrictocin, phenomycin, and enomycin.

- Any method known in the art for conjugating the antibody molecules of the invention to the various moieties may be employed, including those methods  
30 described by Hunter, *et al.*, (1962) Nature 144: 945; David, *et al.*, (1974) Biochemistry 13: 1014; Pain, *et al.*, (1981) J. Immunol. Meth. 40: 219; and Nygren, J., (1982) Histochem. and Cytochem. 30: 407.

Methods for conjugating antibodies are conventional and very well known in the art.

### Screening Assays

The invention allows the identification of selective ligands of NPC1L1 (e.g., SEQ ID NO: 2, 4 or 12) that may be useful in treatment and management of a variety of medical conditions, including elevated serum sterol (e.g., cholesterol) or 5 $\alpha$ -stanol. Thus, NPC1L1 of this invention can be employed in screening systems to identify ligands. These ligands may be agonists or antagonists of NPC1L1. Essentially, these assays provide methods for identifying ligands of NPC1L1 by using (1) NPC1L1, (2) an appropriate known NPC1L1 ligand, agonist or antagonist, for example, a sterol (such as cholesterol, phytosterols, including, but not limited to, sitosterol, campesterol, stigmasterol and avenosterol), a cholesterol oxidation product, a 5 $\alpha$ -stanol (including, but not limited to, cholestanol, 5 $\alpha$ -campestanol and 5 $\alpha$ -sitostanol), a substituted azetidinone (e.g., ezetimibe), BODIPY-ezetimibe (Altmann, *et al.*, (2002) *Biochim. Biophys. Acta* 1580(1): 77-93) or 4", 6"-bis[(2-fluorophenyl)carbamoyl]-beta-D-cellobiosyl derivative of 11-ketotigogenin as described in DeNinno, *et al.*, (1997) (*J. Med. Chem.* 40(16): 2547-54) or any substituted azetidinone, and (3) a sample to be tested for the presence of a candidate NPC1L1 ligand.

The term "specific" when used to describe binding of, for example, a ligand of NPC1L1 in a screening assay is a term of art which refers to the extent by which the ligand or antagonist (e.g., substituted azetidinone, ezetimibe, sterol (such as cholesterol) or 5 $\alpha$ -stanol) binds preferentially to NPC1L1 in comparison to other proteins in the assay system. For example, detection of the specific binding of a ligand of NPC1L1 binds specifically to NPC1L1 is made apparent when a signal generated in the assay to indicate such binding exceeds, to any extent, a signal generated in a negative control wherein, for example, NPC1L1 or ligand is absent. Furthermore, "specific binding" includes binding of a ligand either directly to NPC1L1 or indirectly, for example via another moiety, in a complex of which NPC1L1 is a part. The moiety to which an NPC1L1 ligand binds can be another protein or a post-translational modification of NPC1L1 (e.g., a lipid chain or a carbohydrate chain).

Non-limiting examples of suitable substituted azetidinones for use in the screening assays include those disclosed in U.S. Patent Nos. RE37,721; 5,631,365; 5,767,115; 5,846,966; 5,688,990; 5,656,624; 5,624,920; 5,698,548; 5,756,470; 5,688,787; 5,306,817; 5,633,246; 5,627,176; 5,688,785; 5,744,467; 5 5,846,966; 5,728,827; 6,632,933 and U.S. Patent Application Publication No 2003/0105028—each of which is herein incorporated by reference in its entirety.

The present invention provides for a method by which to evaluate whether a sample contains an NPC1L1 ligand by determining whether the sample contains a candidate compound which competes for binding between the known 10 ligand (*e.g.*, ezetimibe, ezetimibe-glucuronide, compound 2, etc.) and NPC1L1. The ligand may be an agonist or antagonist. In an embodiment of the invention, the binding of the known ligand (*e.g.*, ezetimibe, ezetimibe-glucuronide, compound 2, etc.) to NPC1L1 is disrupted. The term “known ligand” refers to a compound which is known to bind to NPC1L1 and which can be detectably labeled for use in the 15 screening assays and methods described herein. “Known ligands” include the substituted 2-azetidinone glucuronides which can be detectably labeled for use in screening assays as described herein.

Ezetimibe can be prepared by a variety of methods well known to those skilled in the art, for example such as are disclosed in U.S. Patents Nos. 5,631,365, 20 5,767,115, 5,846,966, 6,207,822, U.S. Patent Application Publication No. 2002/0193607 and PCT Patent Application WO 93/02048, each of which is incorporated herein by reference in its entirety.

“Sample”, “candidate compound” or “candidate substance” refers to a compound or composition which is evaluated in a test or assay, for example, for the 25 ability to bind to NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12) or a functional fragment thereof. The composition may comprise candidate compounds, such as small molecules, peptides, nucleotides, polynucleotides, subatomic particles (*e.g.*,  $\alpha$  particles,  $\beta$  particles) or antibodies.

The present invention provides methods for identifying ligands of a 30 compound that binds to NPC1L1 which involve contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and a candidate compound, and determining whether the candidate compound binds to NPC1L1, wherein binding of said candidate compound to NPC1L1 modulates

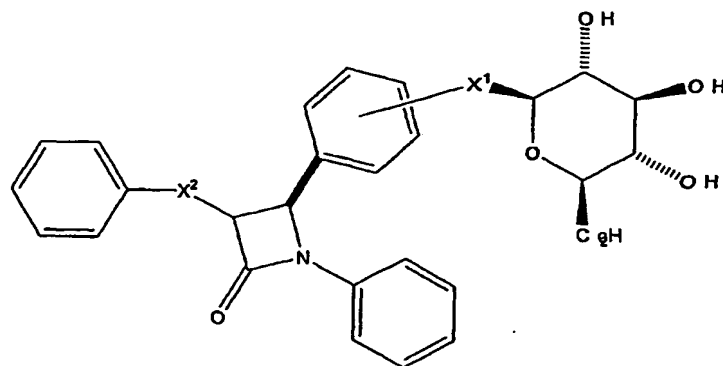


binding of the detectably labeled substituted 2-azetidinone to NPC1L1. The modulation of the binding of the substituted 2-azetidinone to NPC1L1 by the binding of the candidate compound to NPC1L1 indicates that the candidate compound is a ligand that binds to NPC1L1. It is also a good indication that the candidate compound may be an inhibitor of sterol and 5 $\alpha$ -stanol absorption in vivo.

The present invention also provides a method for identifying a ligand of NPC1L1 comprising contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and measuring the binding of NPC1L1 of the detectably labeled substituted 2-azetidinone in the presence and absence of a candidate compound, wherein decreased binding of the detectably labeled substituted 2-azetidinone to the NPC1L1 in the presence of the candidate compound indicates that said candidate compound is a ligand of NPC1L1 and is an inhibitor of sterol and 5 $\alpha$ -stanol absorption.

The substituted 2-azetidinone is detectably labeled with  $^3\text{H}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$ , or a fluorescently labeled substituted 2-azetidinone. Preferably, the substituted 2-azetidinone is labeled with  $^{35}\text{S}$  or  $^{125}\text{I}$ , and particularly  $^{35}\text{S}$ .

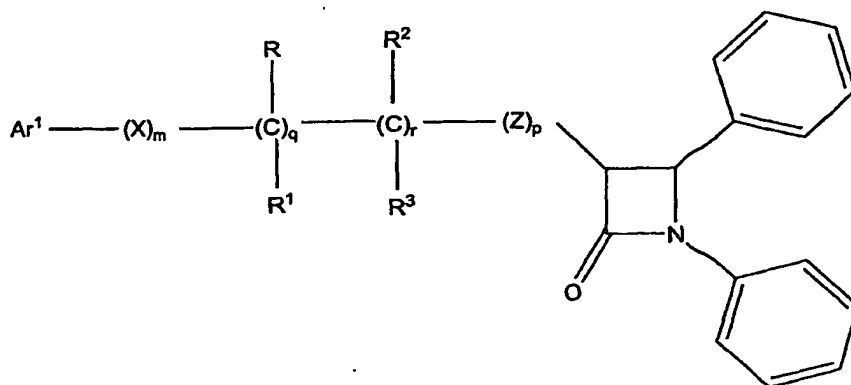
Preferably, the substituted 2-azetidinone is substituted 2-azetidinone-glucuronide. Compounds that are substituted 2-azetidinone-glucuronides are those having the following structure (I):



(I)

wherein  $X^1$  represents a group that links the glucuronide to the 4-phenyl ring, for example but not limited to  $-\text{O}-$  or  $-\text{C}_{1-3}$  alkyl-,  $X^2$  represents an optionally substituted  $-\text{alkanediy}-$ , and wherein any of the phenyl groups may be optionally substituted. Examples of the phenyl- $X^2$ -moiety in structure (I) include

- those represented at the 4-position on the 2-azetidinone structure shown below in structure (II). Additional examples of substituted 2-azetidinone-glucuronides include but are not limited to those described in U.S. Patent No. 5,756,470, WO02/066464 and US 2002/0137689. Additional examples of substituted 2-azetidinone-glucuronide compounds include those having the structure (II) and pharmaceutically acceptable salts and esters thereof as follows:



10

(II)

wherein:

$Ar^1$  is selected from the group consisting of aryl and  $R^4$ -substituted aryl;

$X$ ,  $Y$  and  $Z$  are independently selected from the group consisting of  $-CH_2-$ ,  $-CH(C_{1-6}alkyl)-$  and  $-C(C_{1-6}alkyl)_2-$ ;

$R$  is selected from the group consisting of  $-OR^6$ ,  $-O(CO)R^6$ ,  $-O(CO)OR^9$ ,  $-O(CO)NR^6R^7$ , a sugar residue, a disugar residue, a trisugar residue and a tetrasugar residue;

$R^1$  is selected from the group consisting of  $-H$ ,  $-C_{1-6}alkyl$  and aryl, or  $R$  and  $R^1$  together are oxo;

$R^2$  is selected from the group consisting of  $-OR^6$ ,  $-O(CO)R^6$ ,  $-O(CO)OR^9$  and  $-O(CO)NR^6R^7$ ;

$R^3$  is selected from the group consisting of  $-H$ ,  $-C_{1-6}alkyl$  and aryl or  $R^2$  and  $R^3$  together are oxo;

q, r and t are each independently selected from 0 and 1;

m, n and p are each independently selected from 0, 1, 2, 3 and 4;

R<sup>4</sup> is 1-5 substituents independently selected at each occurrence from the group consisting of:

-OR<sup>5</sup>, -O(CO)R<sup>5</sup>, -O(CO)OR<sup>8</sup>, -O-C<sub>1-5</sub>alkyl-OR<sup>5</sup>, -O(CO)NR<sup>5</sup>R<sup>6</sup>, -NR<sup>5</sup>R<sup>6</sup>, -NR<sup>5</sup>(CO)R<sup>6</sup>, -NR<sup>5</sup>(CO)OR<sup>8</sup>, -NR<sup>5</sup>(CO)NR<sup>6</sup>R<sup>7</sup>, -NR<sup>5</sup>SO<sub>2</sub>R<sup>8</sup>, -COOR<sup>5</sup>, -CONR<sup>5</sup>R<sup>6</sup>, -COR<sup>5</sup>, -SO<sub>2</sub>NR<sup>5</sup>R<sup>6</sup>, -S(O)<sub>t</sub>R<sup>8</sup>, -O-C<sub>1-10</sub>alkyl-COOR<sup>5</sup>, -O-C<sub>1-10</sub>alkyl-CONR<sup>5</sup>R<sup>6</sup> and fluoro;

R<sup>5</sup>, R<sup>6</sup> and R<sup>7</sup> are independently selected at each occurrence from the group consisting of -H, C<sub>1-6</sub>alkyl, aryl and aryl-substituted C<sub>1-6</sub>alkyl;

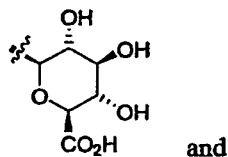
R<sup>8</sup> is independently selected from the group consisting of C<sub>1-6</sub>alkyl, aryl and aryl-substituted C<sub>1-6</sub>alkyl;

R<sup>9</sup> is selected from the group consisting of -C≡C-CH<sub>2</sub>-NR<sup>10</sup>R<sup>11</sup>, -C≡C-C(O)R<sup>13</sup>, and -(CH<sub>2</sub>)<sub>3</sub>-NR<sup>10</sup>R<sup>14</sup>;

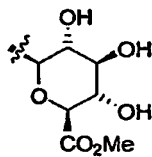
R<sup>10</sup> is independently selected at each occurrence from -H and -C<sub>1-3</sub>alkyl;

R<sup>11</sup> is selected from the group consisting of -H, -C<sub>1-3</sub>alkyl, -C(O)-C<sub>1-3</sub>alkyl, -C(O)-NR<sup>10</sup>R<sup>10</sup>, -SO<sub>2</sub>-C<sub>1-3</sub>alkyl, and -SO<sub>2</sub>-phenyl; and

R<sup>12</sup> is selected from



and



(referred to herein as "glucuronide")

(referred to herein as "methyl ester

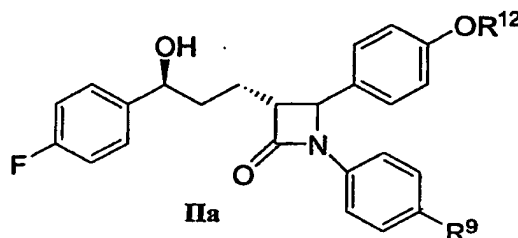
glucuronide");

R<sup>13</sup> is selected from the group consisting of -OH and -NR<sup>10</sup>R<sup>11</sup>; and

R<sup>14</sup> is selected from the group consisting of -C(O)-C<sub>1-3</sub>alkyl, -C(O)-NR<sup>10</sup>R<sup>10</sup>, -SO<sub>2</sub>-C<sub>1-3</sub>alkyl and -SO<sub>2</sub>-phenyl.

In one embodiment of Formula II are compounds wherein q, r and t are each independently selected from 0 and 1; and m, n and p are each independently selected from 0, 1, 2, 3 and 4; provided that at least one of q and r is 1, and the sum of

m, n, p, q are r is 1, 2, 3, 4, 5 or 6; and provided that when p is 0 and r is 1, the sum of m, q and n is 1, 2, 3, 4, or 5. In a second embodiment of Formula II are compounds of Formula IIa,



- 5 In a class of each of these embodiments are compounds wherein R<sup>9</sup> is -C≡C-CH<sub>2</sub>-NR<sup>10</sup>R<sup>11</sup>. In another class of each of these embodiments are compounds wherein R<sup>9</sup> contains an -SO<sub>2</sub>- group, i.e., wherein R<sup>9</sup> is selected from the group consisting of -C≡C-CH<sub>2</sub>-NR<sup>10</sup>R<sup>11</sup>, -C≡C-C(O)NR<sup>10</sup>R<sup>11</sup>, -(CH<sub>2</sub>)<sub>3</sub>-NR<sup>10</sup>-SO<sub>2</sub>-C<sub>1-3</sub>alkyl and -(CH<sub>2</sub>)<sub>3</sub>-NR<sup>10</sup>-SO<sub>2</sub>-phenyl, and R<sup>11</sup> is selected from -SO<sub>2</sub>-C<sub>1-3</sub>alkyl, and -SO<sub>2</sub>-phenyl.

The term "alkyl" is intended to include both branched- and straight-chain saturated aliphatic univalent hydrocarbon groups having the specified number of carbon atoms. Examples of alkyl groups include, but are not limited to, methyl (Me), ethyl (Et), n-propyl (Pr), n-butyl (Bu), n-pentyl, n-hexyl, and the isomers thereof such as isopropyl (i-Pr), isobutyl (i-Bu), secbutyl (s-Bu), tertbutyl (t-Bu), isopentyl, isohexyl and the like. If there is no specified prefix (such as "n-" for normal, "s-" for sec, "t-" for tert, "i-" for iso) with a named alkyl group, then it is intended that the named alkyl group is an n-alkyl group (i.e., "propyl" is "n-propyl"). The term "aryl" is intended to include phenyl (Ph), naphthyl, indenyl, tetrahydronaphthyl or indanyl. Phenyl is preferred.

Suitable protecting groups (designated as "PG" herein) for the hydroxyl groups of R<sup>12</sup> when R<sup>12</sup> is a glucuronide or methyl ester glucuronide include but are not limited to those that are known to be useful as carbohydrate protecting groups, such as for example benzyl, acetyl, benzoyl, *tert*-butyldiphenylsilyl, trimethylsilyl, *para*-methoxybenzyl, benzylidene, and methoxy methyl. Conditions required to selectively add and remove such protecting groups are

found in standard textbooks such as Greene, T, and Wuts, P. G. M., *Protective Groups in Organic Synthesis*, John Wiley & Sons, Inc., New York, NY, 1999.

Compounds of Formula II may contain one or more asymmetric centers and can thus occur as racemates and racemic mixtures, single enantiomers, 5 enantiomeric mixtures, diastereomeric mixtures and individual diastereomers, and all such isomeric forms are within the scope of Formula II.

Radioactive isotopes of the compounds of Formula II are particularly useful in such assays, for example compounds of Formula II wherein sulfur is replaced with "hot"  $^{35}\text{S}$ -, and particularly wherein the radioactive sulfur isotope is 10 incorporated within the  $\text{R}^9$  moiety. The use of all such radioactive isotopes of the compounds of Formula II in an assay for identifying NPC1L1 ligands is included within the scope of this invention.

The term "pharmaceutically acceptable salts" means non-toxic salts of the compounds of Formula II which are generally prepared by reacting the free acid 15 with a suitable organic or inorganic base, particularly those formed from cations such as sodium, potassium, aluminum, calcium, lithium, magnesium, zinc and tetramethylammonium, as well as those salts formed from amines such as ammonia, ethylenediamine, N-methylglucamine, lysine, arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chlorprocaine, diethanolamine, procaine, 20 N-benzylphenethylamine, 1-p-chlorobenzyl-2-pyrrolidine-1'-yl-methylbenzimidazole, diethylamine, piperazine, morpholine, 2,4,4-trimethyl-2-pentamine and tris(hydroxymethyl)aminomethane.

When the compounds of Formula II are basic, salts may be prepared from pharmaceutically acceptable non-toxic acids, including inorganic and organic 25 acids. Such acids include acetic, benzenesulfonic, benzoic, camphorsulfonic, citric, ethanesulfonic, fumaric, gluconic, glutamic, hydrobromic, hydrochloric, isethionic, lactic, maleic, malic, mandelic, methanesulfonic, mucic, nitric, pamoic, pantothenic, phosphoric, succinic, sulfuric, tartaric, p-toluenesulfonic acid, and the like. Particularly preferred are citric, hydrobromic, hydrochloric, maleic, phosphoric, 30 sulfuric, and tartaric acids.

Examples of pharmaceutically acceptable esters include, but are not limited to,  $\text{-C}_{1-4}$  alkyl and  $\text{-C}_{1-4}$  alkyl substituted with phenyl, dimethylamino and acetylamino. " $\text{C}_{1-4}$  alkyl" herein includes straight or branched aliphatic chains

containing from 1 to 4 carbon atoms, for example methyl, ethyl, n-propyl, n-butyl, iso-propyl, sec-butyl and tert-butyl.

The compounds of structural Formula II can be prepared according to the procedures of the following Scheme using appropriate materials, and are further exemplified by specific examples which follow. A variety of chromatographic techniques may be employed in the preparation of the compounds of Formula II. These techniques include, but are not limited to: High Performance Liquid Chromatography (including normal- reversed- and chiral-phase); Super Critical Fluid Chromatography; preparative Thin Layer Chromatography; flash chromatography with silica gel or reversed-phase silica gel; ion-exchange chromatography; and radial chromatography. All temperatures are degrees Celsius unless otherwise noted.

Some abbreviations used herein include:

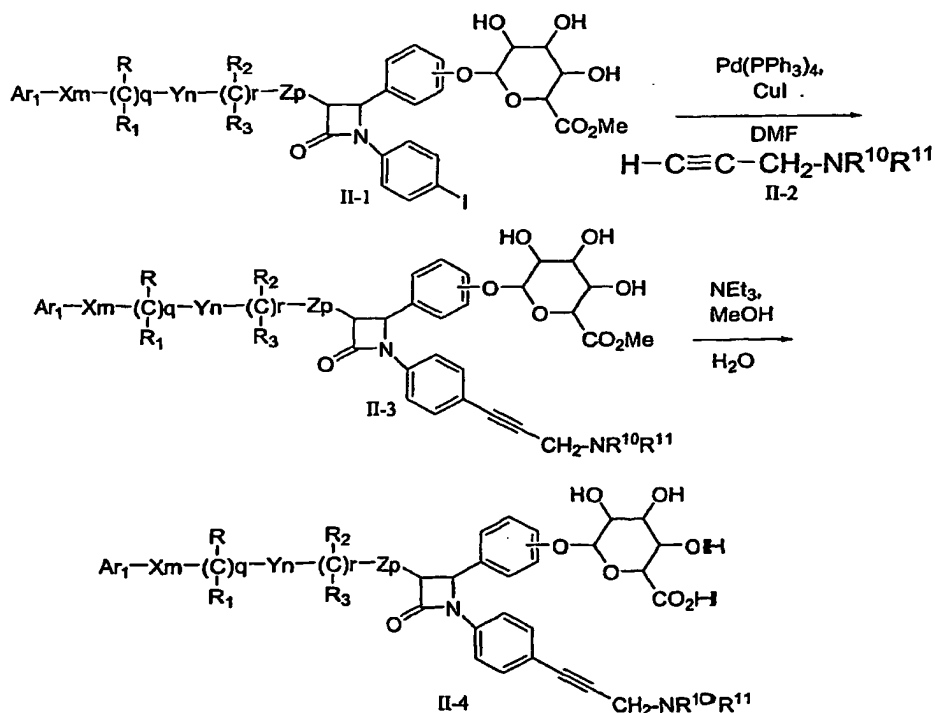
Ac	Acyl (CH <sub>3</sub> C(O)-)
Bn	benzyl
calc.	Calculated
Celite	Celite™ diatomaceous earth
Dess-Martin Periodinane	1,1,1-tris(acetyloxy)-1,1-dihydro-1,2-benzodioxol-3-(1H)-one
DMF	N,N-dimethylformamide
equiv.	Equivalent(s)
ES-MS	Electron Spray Ion-Mass Spectroscopy
EtOAc	Ethyl acetate
h	Hour(s)
HPLC	High performance liquid chromatography
min	Minute(s)
m.p.	Melting point
MS	Mass spectrum
r.t. (or rt)	Room temperature
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Tlc	Thin layer chromatography

The general Scheme below illustrates a method for the syntheses of compounds of structural formula II-4. All substituents are as defined in Formula II unless indicated otherwise. In this method, II-1 is treated with a terminal alkyne of

type II-2 in the presence of a suitable palladium catalyst such as tetrakis(triphenylphosphine) palladium(0) or [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II) or the like, and copper(I) iodide. The reaction is usually performed in an inert organic solvent such as DMF, between room temperature and 100 °C, for a period of 6-48 h, and the product is an internal alkyne of structural formula II-3. Alkyne II-2 may contain a radioactive atom such as <sup>35</sup>S to provide the corresponding radiolabeled adduct upon reaction with II-1. Conversion of II-3 to II-4 can be achieved using a variety of hydrolytic methods known to those skilled in the art of organic synthesis. For example, a particularly mild hydrolysis protocol involves the treatment of II-3 with a tertiary amine base such as triethylamine, or diisopropylethylamine or the like, in a mixed solvent system comprising methanol and water. The product of the reaction is a compound of structural formula II-4. By utilizing the procedures described herein, one of ordinary skill in the art can readily prepare additional compounds of Formula II.

15

## SCHEME



Two additional types of screening systems that can be used include a labeled-ligand binding assay (*e.g.*, direct binding assay or scintillation proximity assay (SPA)) and a "sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol uptake" assay. A labeled ligand, for use in the binding assay, can be obtained by labeling a sterol (*e.g.*,  
5 cholesterol) or a 5 $\alpha$ -stanol or a known NPC1L1 agonist or antagonist with a measurable group (*e.g.*,  $^{35}\text{S}$ ,  $^{125}\text{I}$  or  $^3\text{H}$ ). Various labeled forms of sterols (*e.g.*, cholesterol) or 5 $\alpha$ -stanols are available commercially or can be generated using standard techniques (*e.g.*, Cholesterol- [1,2- $^3\text{H}$ (N)], Cholesterol-[1,2,6,7- $^3\text{H}$ (N)] or Cholesterol-[7- $^3\text{H}$ (N)]; American Radiolabeled Chemicals, Inc; St. Louis, MO). In a  
10 preferred embodiment, ezetimibe is fluorescently labeled with a BODIPY group (Altmann, *et al.*, (2002) *Biochim. Biophys. Acta* 1580(1): 77-93) or labeled with a detectable group such as  $^{35}\text{S}$ ,  $^{125}\text{I}$  or  $^3\text{H}$ , preferably  $^{35}\text{S}$ .

**Direct Binding Assay.** Typically, a given amount of NPC1L1 of the invention (*e.g.*, SEQ ID NO: 2, 4 or 12) or a complex including NPC1L1 is contacted  
15 with increasing amounts of labeled ligand or known antagonist or agonist (discussed above) and the amount of the bound, labeled ligand or known antagonist or agonist is measured after removing unbound, labeled ligand or known antagonist or agonist by washing. As the amount of the labeled ligand or known agonist or antagonist is increased, a point is eventually reached at which all receptor binding sites are  
20 occupied or saturated. Specific receptor binding of the labeled ligand or known agonist or antagonist is abolished by a large excess of unlabeled ligand or known agonist or antagonist.

Preferably, an assay system is used in which non-specific binding of the labeled ligand or known antagonist or agonist to the receptor is minimal. Non-  
25 specific binding is typically less than 50%, preferably less than 15%, more preferably less than 10%, and most preferably 5% or less, of the total binding of the labeled ligand or known antagonist or agonist.

In the basic binding assay, the method for identifying an NPC1L1 ligand, agonist or antagonist includes:

- 30 (a) contacting NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12), a fragment thereof or a complex including NPC1L1, in the presence of a known amount of labeled sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol or known antagonist or agonist (*e.g.*,



labeled ezetimibe) with a sample to be tested for the presence of an NPC1L1 ligand, agonist or antagonist; and

(b) measuring the amount of labeled sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol or known antagonist or agonist directly or indirectly bound to NPC1L1.

5 An NPC1L1 ligand in the sample is identified by measuring substantially reduced direct or indirect binding of the labeled sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol or known antagonist or agonist to NPC1L1, compared to what would be measured in the absence of such a ligand. For example, reduced direct or indirect binding between [ $^3$ H]-cholesterol and NPC1L1 in the presence of a sample might  
10 suggest that the sample contains a substance which is competing against [ $^3$ H]-cholesterol for NPC1L1 binding.

This assay can include a control experiment lacking any NPC1L1-dependent ligand (*e.g.*, sterol such as cholesterol or 5 $\alpha$ -stanol) binding. In this assay, for example, a whole cell or cell membrane lacking any functional NPC1L1, for  
15 example, a cell or membrane isolated or derived from a transgenic mutant *npc1l1*<sup>-/-</sup> mouse of the invention, is assayed for ligand binding. When screening a sample for the presence of an NPC1L1 antagonist, it is useful to compare the level of binding observed in the presence of a sample being tested with that of a control experiment, as described herein, which completely lacks NPC1L1-dependent binding. Ideally,  
20 though by no means necessarily, the level of binding seen in the presence of a sample containing an antagonist will be similar to that of the control experiment.

Alternatively, a sample can be tested directly for binding to NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12). A basic assay of this type may include the following steps:

25 (a) contacting NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12), a fragment thereof or a complex including NPC1L1 with a labeled candidate compound (*e.g.*, [ $^3$ H]-ezetimibe); and

(b) detecting direct or indirect binding between the labeled candidate compound and NPC1L1.

30 Again, these experiment can be performed along with a control experiment wherein NPC1L1-dependent binding is completely lacking. For example, the assay can be performed using a whole cell or cell membrane lacking any

functional NPC1L1 (e.g., cell or cell membrane derived from a transgenic, mutant *npc1l1*<sup>-/-</sup> mouse as described herein).

A candidate compound which is found to bind to NPC1L1 may function as ligand, agonist or antagonist of NPC1L1 (e.g., by inhibition of sterol  
5 (e.g., cholesterol) or 5 $\alpha$ -stanol uptake).

In an embodiment of the invention, the bound candidate compound is quantified after filtration using glass fiber filters. In one aspect of this embodiment, the bound candidate compound is detected after single-tube vacuum filtration of GF/C glass fiber filters, obtained from Whatman. The filters may be pretreated by soaking  
10 with 0.5% polyethylenimine to reduce nonspecific binding. Filtration is accomplished by adding ice cold buffer to the assay tube, pouring the mixture through the filter, and then rinsing the tube and filter twice more with additional buffer. The buffer may be a Tris buffer or MES buffer (120 mM NaCl, 0.1% sodium cholate, and 20 mM MES at pH 6.70). The filters can be counted using scintillation fluid, e.g., Packard DM  
15 liquid or Packard Ultima Gold MV.

Alternatively, vacuum filtration of the sample on a Millipore 96-well plate (Whatman GF/C) can also be used to achieve adequate precision in a manner well-known to those skilled in the art.

*SPA Assay.* NPC1L1 ligands may also be measured using scintillation  
20 proximity assays (SPA). SPA assays are conventional and very well known in the art; see, for example, U.S. Patent No. 4,568,649. In SPA, the target of interest is immobilized to a small microsphere approximately 5 microns in diameter. The microsphere, typically, includes a solid scintillant core which has been coated with a polyhydroxy film, which in turn contains coupling molecules, which allow generic  
25 links for assay design. When a radioisotopically labeled molecule binds to the microsphere, the radioisotope is brought into close proximity to the scintillant and effective energy transfer from electrons emitted by the isotope will take place resulting in the emission of light. While the radioisotope remains in free solution, it is too distant from the scintillant and the electron will dissipate the energy into the  
30 aqueous medium and therefore remain undetected. Scintillation may be detected with a scintillation counter. In general, <sup>3</sup>H, <sup>125</sup>I and <sup>35</sup>S labels are well suited to SPA.

For the assay of receptor-mediated binding events, the lectin wheat germ agglutinin (WGA) may be used as the SPA bead coupling molecule (Amersham

Biosciences; Piscataway, NJ). The WGA coupled bead captures glycosylated, cellular membranes and glycoproteins and has been used for a wide variety of receptor sources and cultured cell membranes. The receptor is immobilized onto the WGA-SPA bead and a signal is generated on binding of an isotopically labeled  
5 ligand. Other coupling molecules which may be useful for receptor binding SPA assays include poly-L-lysine and WGA/polyethyleneimine (Amersham Biosciences; Piscataway, NJ). See, for example, Berry, J.A., *et al.*, (1991) Cardiovascular Pharmacol. 17 (Suppl.7): S143-S145; Hoffman, R., *et al.*, (1992) Anal. Biochem. 203: 70-75; Kienhus, *et al.*, (1992) J. Receptor Research 12: 389-399; Jing, S., *et al.*,  
10 (1992) Neuron 9: 1067-1079.

The scintillant contained in SPA beads may include, for example, yttrium silicate (YSi), yttrium oxide (YOx), diphenyloxazole or polyvinyltoluene (PVT) which acts as a solid solvent for diphenylanthracene (DPA).

SPA assays may be used to analyze whether a sample contains an  
15 NPC1L1 ligand. In these assays, a host cell which expresses NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12) on the cell surface or a membrane fraction thereof is incubated with and captured by SPA beads (*e.g.*, WGA coated YOx beads or WGA coated YSi beads). The beads bearing the NPC1L1 are incubated with labeled, known ligand or agonist or antagonist (*e.g.*, <sup>3</sup>H-cholesterol, <sup>3</sup>H-ezetimibe, <sup>125</sup>I-ezetimibe or a <sup>35</sup>S-  
20 ezetimibe analog). The assay mixture further includes either the sample to be tested or a blank (*e.g.*, water). After an optional incubation, scintillation is measured using a scintillation counter. An NPC1L1 ligand, agonist or antagonist may be identified in the sample by measuring substantially reduced fluorescence, compared to what would be measured in the absence of such ligand, agonist or antagonist (blank). Measuring  
25 substantially reduced fluorescence may suggest that the sample contains a substance which competes for direct or indirect NPC1L1 binding with the known ligand, agonist or antagonist.

Alternatively, a sample may be identified as an ligand of NPC1L1 by directly detecting binding in a SPA assay. In this assay, a labeled version of a  
30 candidate compound to be tested may be put in contact with the host cell expressing NPC1L1 or a membrane fraction thereof which is bound to the SPA bead. Fluorescence may then be assayed to detect the presence of a complex between the labeled candidate compound and the host cell or membrane fraction expressing

NPC1L1 or a complex including NPC1L1. A candidate compound which binds directly or indirectly to NPC1L1 may possess NPC1L1 agonistic or antagonistic activity.

SPA Assays can also be performed along with a control experiment lacking any NPC1L1-dependent binding. The control experiment can be performed, for example, with a cell or cell membrane lacking any functional NPC1L1 (e.g., cell or cell membrane derived from a transgenic, mutant *npc1l1*- mouse as described herein). When the control experiment is performed, the level of binding observed in the presence of sample being tested for the presence of an antagonist can be compared with that observed in the control experiment.

**Sterol/5 $\alpha$ -stanol Uptake Assay.** Assays may also be performed to determine if a sample can agonize or antagonize NPC1L1 mediated sterol (e.g., cholesterol) or 5 $\alpha$ -stanol uptake. In these assays, a host cell expressing NPC1L1 (e.g., SEQ ID NO: 2 or 4 or 12) on the cell surface (discussed above) can be contacted with detectably labeled sterol (e.g., <sup>3</sup>H-cholesterol or <sup>125</sup>I-cholesterol) or 5 $\alpha$ -stanol along with either a sample or a blank. After an optional incubation, the cells can be washed to remove unabsorbed sterol or 5 $\alpha$ -stanol. Sterol or 5 $\alpha$ -stanol uptake can be determined by detecting the presence of labeled sterol or 5 $\alpha$ -stanol in the host cells. For example, assayed cells or lysates or fractions thereof (e.g., fractions resolved by thin-layer chromatography) can be contacted with a liquid scintillant and scintillation can be measured using a scintillation counter.

In these assays, an NPC1L1 antagonist in the sample may be identified by measuring substantially reduced uptake of labeled sterol (e.g., <sup>3</sup>H-cholesterol) or 5 $\alpha$ -stanol, compared to what would be measured in the absence of such an antagonist and an agonist may be identified by measuring substantially increased uptake of labeled sterol (e.g., <sup>3</sup>H-cholesterol) or 5 $\alpha$ -stanol, compared to what would be measured in the absence of such an agonist.

Uptake assays can also be performed along with a control experiment lacking any NPC1L1-dependent uptake. The control experiment can be performed, for example, with a cell lacking any functional NPC1L1 (e.g., cell derived from a transgenic, mutant *npc1l1*- mouse as described herein). When the control experiment is performed, the level of uptake observed in the presence of sample being tested for

the presence of an antagonist can be compared with that observed in the control experiment.

*Source of NPC1L1.* In principle, a binding assay of the invention could be carried out using a soluble NPC1L1 polypeptide of the invention, *e.g.*,  
5 following production and refolding by standard methods from an *E. coli* or other prokaryotic or eukaryotic expression system, and the resulting receptor-labeled ligand complex could be precipitated, *e.g.*, using an antibody against the receptor. The precipitate could then be washed and the amount of the bound, labeled ligand or antagonist or agonist could be measured.

10 Alternatively, NPC1L1 is membrane-bound. A nucleic acid encoding an NPC1L1 polypeptide of the invention (*e.g.*, SEQ ID NO: 2, 4 or 12) can be transfected into an appropriate host cell, whereby the NPC1L1 will become incorporated into the membrane of the cell. A membrane fraction can then be isolated from the cell and used as a source of NPC1L1 for assay. Alternatively, the whole cell  
15 expressing NPC1L1 in the cell surface can be used in an assay. Preferably, specific binding of the labeled ligand or known antagonist or agonist to an untransfected/untransformed host cell or to a membrane fraction from an untransfected/untransformed host cell will be negligible.

Various membranes may be used directly as a source of NPC1L1 for  
20 the above-described screening systems, *e.g.* direct binding, scintillation proximity assay, sterol/5 $\alpha$  stanol uptake assay. As described in Examples 5, 6, 7, 8, 9, 17, 27, and 29, NPC1L1 is highly expressed in certain tissues, especially in brush border cells of intestinal tissues. Therefore, brush border membrane (BBM) vesicle preparations may be utilized as a source of NPC1L1. The membranes may be derived from  
25 mammalian intestinal tissue from rhesus, rat, mouse or human tissue.

Membranes may be derived from brush border cells of intestinal tissues. Such membranes are conventionally prepared by collecting intestinal tissue from freshly sacrificed animals. The mucosa of the tissue is scraped, collected into buffered solutions, and homogenized. Cellular debris is removed and the membrane  
30 fractions are collected by centrifugation. Conventional techniques known to one of skill in the art may be used for the preparation of brush border membrane vesicles. See Hauser, H., Howell, K., Dawson, R.M.C., Bowyer, D. E. *Biochim. Biophys. Acta* 602, 567-577 (1980); Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal,

K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. 268, 18035-18046 (1993);

Rigtrup, K.M., Ong, D.E. Biochemistry 31, 2920-2926 (1992).

The membrane preparation may be in vesicular or non-vesicular form.

Alternatively, liposomes and liposomal preparations comprising

- 5 NPC1L1 may also be a viable source of NPC1L1 for the screening assays of the present claimed method.

- In vitro* cultured cells expressing NPC1L1 may also be used. The host cells may be prepared by transforming or transfecting a nucleic acid encoding an NPC1L1 of the invention into an appropriate host cell, whereby the receptor becomes  
10 incorporated into the membrane of the cell. A membrane fraction can then be isolated from the cell and used as a source of the receptor for assay. Alternatively, the whole cell expressing the receptor on the cell surface can be used in an assay. Preferably, specific binding of the labeled ligand or known antagonist or agonist to an untransfected/untransformed host cell or membrane fraction from an  
15 untransfected/untransformed host cell will be negligible.

Preferred host cells include Chinese Hamster Ovary (CHO) cells, murine macrophage J774 cells, HEK-293 cells or any other macrophage cell line and human intestinal epithelial Caco2 cells.

- The present invention provides for a method of identifying a ligand of  
20 NPC1L1 using these membrane preparations, for example by contacting membranes comprising NPC1L1, such as brush border membrane vesicle preparations, with detectably labeled substituted azetidinone compounds which are known NPC1L1 ligands, agonists or antagonists, and a candidate compound and determining whether the candidate compound can bind to NPC1L1. The binding of the candidate  
25 compound to NPC1L1 may modulate binding of the detectably labeled NPC1L1 ligands, agonists or antagonists to NPC1L1. In addition, a NPC1L1 ligand may be identified by measuring the binding of NPC1L1 with detectably labeled NPC1L1 ligands, agonists or antagonists in the presence and absence of the candidate compound wherein decreased binding of the detectably labeled NPC1L1 ligands,  
30 agonists or antagonists to NPC1L1 is an indication that the candidate compound is ligand of NPC1L1.

NPC1L1 may also be obtained by solubilization of membrane fractions comprising NPC1L1. The membranes may be obtained as discussed above, *e.g.*, from mammalian tissue or *in vitro* cultured cells.

**Binding Affinities of NPC1L1 Ligands.** The affinity and specificity of the known ligand (*e.g.*, detectably labeled substituted 2-azetidinone-glucuronide) for NPC1L1 are important to the identification of ligands that bind NPC1L1 in a screening assay. It is understood that the known ligand will be labeled for use in the screening assay. In an embodiment of the invention, the binding affinity of the known ligand for human NPC1L1 has a  $K_D$  value equivalent or lower than the  $K_D$  value of ezetimibe glucuronide **1** for human NPC1L1. In an aspect of this embodiment, the binding affinity of the known ligand for human NPC1L1 has a  $K_D$  value of about 200nM or lower; particularly it has a  $K_D$  value of about 100nM or lower; more particularly it has a  $K_D$  value of about 50nM or lower; even more particularly it has a  $K_D$  value of about 20nM or lower; and most particularly it has a  $K_D$  value of about 10nM or lower. For usefulness in the assay, there is essentially no lower limit on the  $K_D$  value of the known ligand and it may, for example, go down into the pM range. As the  $K_D$  value decreases, the binding affinity of the ligand for human NPC1L1 increases, which is desirable for the screening assay.

In another embodiment of the invention, the binding affinity of the known ligand for rat NPC1L1 has a  $K_D$  value equivalent or lower than the  $K_D$  value of ezetimibe glucuronide **1** for rat NPC1L1. In an aspect of this embodiment, the binding affinity of the known ligand for rat NPC1L1 has a  $K_D$  value of about 200nM or lower; particularly it has a  $K_D$  value of about 100nM or lower; more particularly it has a  $K_D$  value of about 50nM or lower; even more particularly it has a  $K_D$  value of about 20nM or lower; and most particularly it has a  $K_D$  value of about 10nM or lower.

In another embodiment of this invention, the known ligand for human NPC1L1 is selected from (a) a sulfur-containing substituted 2-azetidinone-glucuronide that is labeled with  $^{35}\text{S}$ , and particularly a compound of Formula II wherein  $\text{R}^9$  contains an  $-\text{SO}_2-$  group and (b) a substituted 2-azetidinone-glucuronide labeled with  $^{125}\text{I}$ .

In one aspect of this embodiment, the known ligand for human NPC1L1 is selected from (a) a sulfur-containing substituted 2-azetidinone-glucuronide that is labeled with  $^{35}\text{S}$ , and particularly a compound of Formula II

wherein R<sup>9</sup> contains an -SO<sub>2</sub>- group, and (b) a substituted 2-azetidinone-glucuronide labeled with <sup>125</sup>I, and has a K<sub>D</sub> value equivalent or lower than the K<sub>D</sub> value of ezetimibe glucuronide 1.

In another aspect of this embodiment, the known ligand for human  
5 NPC1L1 is selected from (a) a sulfur-containing substituted 2-azetidinone-glucuronide that is labeled with <sup>35</sup>S, and particularly a compound of Formula II wherein R<sup>9</sup> contains an -SO<sub>2</sub>- group, and (b) a substituted 2-azetidinone-glucuronide labeled with <sup>125</sup>I, and has a K<sub>D</sub> value of about 200nM or lower; particularly it has a K<sub>D</sub>  
10 value of about 100nM or lower; more particularly it has a K<sub>D</sub> value of about 50nM or lower; even more particularly it has a K<sub>D</sub> value of about 20nM or lower; and most particularly it has a K<sub>D</sub> value of about 10nM or lower.

When using <sup>3</sup>H-labeled ezetimibe glucuronide in screening to identify NPC1L1 ligands from among the candidate compounds using mouse-derived membranes, candidate compounds identified as NPC1L1 ligands are preferably those  
15 candidates that exhibit a binding affinity having a K<sub>D</sub> value of about 12,000nM or lower, preferably about 1000nM or lower, more preferably about 100nM or lower, and most preferably about 10nM or lower. When using <sup>3</sup>H-labeled ezetimibe glucuronide in screening to identify NPC1L1 ligands using rat-derived membranes or human-derived membranes, candidate compounds identified as NPC1L1 ligands are  
20 preferably those candidates that exhibit a binding affinity having a K<sub>D</sub> value of about 1000nM or lower, preferably about 100nM or lower, and more preferably about 10nM or lower. When using <sup>3</sup>H-labeled ezetimibe glucuronide in screening to identify NPC1L1 ligands using rhesus monkey-derived membranes, candidate compounds identified as NPC1L1 ligands are preferably those candidates that exhibit a binding  
25 affinity having a K<sub>D</sub> value of about 50nM or lower, and preferably about 10nM or lower.

When using <sup>35</sup>S-labeled compound 2 in screening to identify NPC1L1 ligands from among the candidate compounds using rat or human-derived membranes, candidate compounds identified as NPC1L1 ligands are preferably those  
30 candidates that exhibit a binding affinity having a K<sub>D</sub> value in the range from about 10μM to about 1nM. When using <sup>125</sup>I-labeled substituted 2-azetidinone glucuronide compounds in the assay with rat or human membranes, candidate compounds identified as NPC1L1 ligands are preferably those candidates that exhibit a binding



affinity having a  $K_D$  value in the range from about 10nM to about 10pM, and preferably from about 100pM to about 10pM.

*Mouse Assay.* The present invention comprises a mutant, transgenic mouse which lacks any functional NPC1L1. This mouse may serve as a convenient control experiment in screening assays for identifying inhibitors of intestinal sterol (e.g., cholesterol) or 5 $\alpha$ -stanol absorption, preferably inhibitors of NPC1L1. Preferably, a mouse assay of the present invention would comprise the following steps:

- (a) feeding a sterol (e.g., cholesterol) or 5 $\alpha$ -stanol-containing substance (e.g., comprising radiolabeled cholesterol, such as  $^{14}\text{C}$ -cholesterol or  $^3\text{H}$ -cholesterol) to a first and second mouse comprising a functional *NPC1L1* gene and to a third, mutant mouse lacking a functional NPC1L1;

The sterol (e.g., cholesterol) or 5 $\alpha$ -stanol containing substance preferably contains labeled cholesterol, such as a radiolabeled cholesterol, for example,  $^3\text{H}$  or  $^{14}\text{C}$  labeled cholesterol. The sterol (e.g., cholesterol) or 5 $\alpha$ -stanol containing substance may also include cold, unlabeled sterol (e.g., cholesterol) or 5 $\alpha$ -stanol such as in corn oil.

In these assays, the third *npc1l1*<sup>-</sup> mutant mouse serves as a (+)-control experiment which exhibits low levels of intestinal sterol (e.g., cholesterol) or 5 $\alpha$ -stanol absorption and the second mouse serves as a (-)-control experiment which exhibits normal, uninhibited levels of intestinal sterol (e.g., cholesterol) or 5 $\alpha$ -stanol absorption. The second mouse is not administered the sample to be tested for an NPC1L1 antagonist. The first mouse is the experiment.

- (b) administering the sample to the first mouse comprising a functional *NPC1L1* but not to the second mouse;

(c) measuring the amount of sterol (e.g., cholesterol) or 5 $\alpha$ -stanol absorption in the intestine of said first, second and third mouse;

Intestinal sterol (e.g., cholesterol) or 5 $\alpha$ -stanol absorption may be measured by any method known in the art. For example, the level intestinal absorption can be assayed by measuring the level of serum sterol (e.g., cholesterol) or 5 $\alpha$ -stanol.

- (d) comparing the levels of intestinal sterol (e.g., cholesterol) or 5 $\alpha$ -stanol absorption in each mouse;

wherein the sample is determined to contain the intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption antagonist when the level of intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption in the first mouse and in the third mouse are less than the amount of intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption in the  
5 second mouse.

Preferably, if the sample contains an intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption inhibitor (*e.g.*, an NPC1L1 inhibitor), the level of sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption in the first mouse will be similar to that of the third, *npc1l1* mutant mouse.

10 An alternative, (+)-control experiment which may be used in these screening assays is a mouse comprising functional NPC1L1 which is administered a known antagonist of NPC1L1, such as ezetimibe.

#### Pharmaceutical Compositions

NPC1L1 ligands discovered, for example, by the screening methods  
15 described above may be used therapeutically (*e.g.*, in a pharmaceutical composition) to stimulate or block the activity of NPC1L1 and, thereby, to treat any medical condition caused or mediated by NPC1L1. In addition, the antibody molecules of the invention may also be used therapeutically (*e.g.*, in a pharmaceutical composition) to bind NPC1L1 and, thereby, block the ability of NPC1L1 to bind a sterol (*e.g.*,  
20 cholesterol) or 5 $\alpha$ -stanol. Blocking the binding of a sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol would prevent absorption of the molecule (*e.g.*, by intestinal cells such as enterocytes). Blocking absorption of sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol would be a useful way to lower serum sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol levels in a subject and, thereby, reduce the incidence of, for example, hyperlipidemia, atherosclerosis,  
25 coronary heart disease, stroke or arteriosclerosis.

The term "subject" or "patient" includes any organism, preferably animals, more preferably mammals (*e.g.*, mice, rats, rabbits, dogs, horses, primates, cats) and most preferably humans.

The term "pharmaceutical composition" refers to a composition  
30 including an active ingredient and a pharmaceutically acceptable carrier and/or adjuvant.

Although the compositions of this invention could be administered in simple solution, they are more typically used in combination with other materials such

as carriers, preferably pharmaceutically acceptable carriers. Useful, pharmaceutically acceptable carriers can be any compatible, non-toxic substances suitable for delivering the compositions of the invention to a subject. Sterile water, alcohol, fats, waxes, and inert solids may be included in a pharmaceutically acceptable carrier.

- 5 Pharmaceutically acceptable adjuvants (buffering agents, dispersing agents) may also be incorporated into the pharmaceutical composition.

Preferably, the pharmaceutical compositions of the invention are in the form of a pill or capsule. Methods for formulating pills and capsules are very well known in the art. For example, for oral administration in the form of tablets or  
10 capsules, the active drug component may be combined with any oral, non-toxic pharmaceutically acceptable inert carrier, such as lactose, starch, sucrose, cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, talc, mannitol, ethyl alcohol (liquid forms) and the like. Moreover, when desired or needed, suitable binders, lubricants, disintegrating agents and coloring agents may also be incorporated  
15 in the mixture. Suitable binders include starch, gelatin, natural sugars, corn sweeteners, natural and synthetic gums such as acacia, sodium alginate, carboxymethylcellulose, polyethylene glycol and waxes. Among the lubricants there may be mentioned for use in these dosage forms, boric acid, sodium benzoate, sodium acetate, sodium chloride, and the like. Disintegrants include starch, methylcellulose,  
20 guar gum and the like. Sweetening and flavoring agents and preservatives may also be included where appropriate.

The pharmaceutical compositions of the invention may be administered in conjunction with a second pharmaceutical composition or substance. In preferred embodiments, the second composition includes a cholesterol-lowering  
25 drug. When a combination therapy is used, both compositions may be formulated into a single composition for simultaneous delivery or formulated separately into two or more compositions (e.g., a kit).

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. See, e.g.,  
30 Gilman *et al.* (eds.) (1990), *The Pharmacological Bases of Therapeutics*, 8th Ed., Pergamon Press; and Remington's *Pharmaceutical Sciences*, *supra*, Easton, Penn.; Avis *et al.* (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications* Dekker, New York; Lieberman *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms*:

*Tablets* Dekker, New York; and Lieberman *et al.* (eds.) (1990), *Pharmaceutical Dosage Forms: Disperse Systems* Dekker, New York.

5 The dosage regimen involved in a therapeutic application may be determined by a physician, considering various factors which may modify the action of the therapeutic substance, *e.g.*, the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration, and other clinical factors. Often, treatment dosages are titrated upward from a low level to optimize safety and efficacy. Dosages may be adjusted to account for the smaller molecular sizes and possibly decreased half-lives (clearance times) following administration.

10 An "effective amount" of a ligand of the invention may be an amount that will detectably reduce the level of intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption or detectably reduce the level of serum sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol in a subject administered the composition.

15 Typical protocols for the therapeutic administration of such substances are well known in the art. Pharmaceutical composition of the invention may be administered, for example, by any parenteral or non-parenteral route.

Pills and capsules of the invention can be administered orally. Injectable compositions can be administered with medical devices known in the art; for example, by injection with a hypodermic needle.

20 Injectable pharmaceutical compositions of the invention may also be administered with a needleless hypodermic injection device; such as the devices disclosed in U.S. Patent Nos. 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556.

#### Anti-Sense

25 The present invention also encompasses anti-sense oligonucleotides capable of specifically hybridizing to mRNA encoding NPC1L1 (*e.g.*, any of SEQ ID NOs: 1, 3, 5-11 or 13) having an amino acid sequence defined by, for example, SEQ ID NO: 2 or 4 or 12 or a subsequence thereof so as to prevent translation of the mRNA. Additionally, this invention contemplates anti-sense oligonucleotides capable  
30 of specifically hybridizing to the genomic DNA molecule encoding NPC1L1, for example, having an amino acid sequence defined by SEQ ID NO: 2 or 4 or 12 or a subsequence thereof.

This invention further provides pharmaceutical compositions comprising (a) an amount of an oligonucleotide effective to reduce NPC1L1-mediated sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption by passing through a cell membrane and binding specifically with mRNA encoding NPC1L1 in the cell so as to prevent its translation and (b) a pharmaceutically acceptable carrier capable of passing through a cell membrane. In an embodiment, the oligonucleotide is coupled to a substance that inactivates mRNA. In another embodiment, the substance that inactivates mRNA is a ribozyme.

Reducing the level of NPC1L1 expression by introducing anti-sense NPC1L1 RNA into the cells of a patient is a useful method reducing intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption and serum cholesterol in the patient.

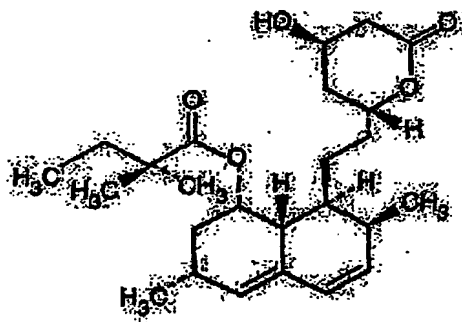
#### Kits

Kits of the present invention include ezetimibe, preferably combined with a pharmaceutically acceptable carrier, in a pharmaceutical formulation, more preferably in a pharmaceutical dosage form such as a pill, a powder, an injectable liquid, a tablet, dispersible granules, a capsule, a cachet or a suppository. See for example, Gilman *et al.* (eds.) (1990), *The Pharmacological Bases of Therapeutics*, 8th Ed., Pergamon Press; and Remington's *Pharmaceutical Sciences*, supra, Easton, Penn.; Avis *et al.* (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications* Dekker, New York; Lieberman *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Tablets* Dekker, New York; and Lieberman *et al.* (eds.) (1990), *Pharmaceutical Dosage Forms: Disperse Systems* Dekker, New York. Preferably, the dosage form is a Zetia® tablet (Merck/Schering-Plough Corp.). Ezetimibe may be supplied in any convenient form. For example, tablets including ezetimibe may be supplied in bottles of 30, 90 or 500.

The kits of the present invention also include information, for example in the form of a package insert, indicating that the target of ezetimibe is NPC1L1 (NPC3). The term "target of ezetimibe" indicates that ezetimibe reduces intestinal sterol (*e.g.*, cholesterol) or 5 $\alpha$ -stanol absorption, either directly or indirectly, by antagonizing NPC1L1. The form of the insert may take any form, such as paper or on electronic media such as a magnetically recorded medium (*e.g.*, floppy disk) or a CD-ROM.

The package insert may also include other information concerning the pharmaceutical compositions and dosage forms in the kit. Generally, such information aids patients and physicians in using the enclosed pharmaceutical compositions and dosage forms effectively and safely. For example, the following information regarding ezetimibe (*e.g.*, Zetia®) and/or simvastatin (*e.g.*, Zocor®) may be supplied in the insert: pharmacokinetics, pharmacodynamics, clinical studies, efficacy parameters, indications and usage, contraindications, warnings, precautions, adverse reactions, overdose, proper dosage and administration, how supplied, proper storage conditions, references and patent information.

10 The kits of the invention may also include simvastatin (



) preferably combined with a pharmaceutically acceptable carrier, in a pharmaceutical formulation, more preferably in a pharmaceutical dosage form such as a pill, a powder, an injectable liquid, a tablet, dispersible granules, a capsule, a cachet or a suppository. Preferably, the dosage form of simvastatin is a Zocor® tablet (Merck & Co.; Whitehouse Station, NJ).

Tablets or pills comprising simvastatin may be supplied in any convenient form. For example, pills or tablets comprising 5mg simvastatin can be supplied as follows: bottles of 30, 60, 90, 100 or 1000. Pills or tablets comprising 10 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000. Pills or tablets comprising 20 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000. Pills or tablets comprising 40 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100 or 1000. Pills or tablets comprising 80 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000.

Ezetimibe and simvastatin may be supplied, in the kit, as separate compositions or combined into a single composition. For example, ezetimibe and simvastatin may be supplied within a single, common pharmaceutical dosage form (e.g., pill or tablet) as in separate pharmaceutical dosage forms (e.g., two separate pills or tablets).

#### ***npc111*<sup>-</sup> Cells**

The present invention provides any isolated mammalian cell, (e.g., an isolated mouse cell, an isolated rat cell or an isolated human cell) which lacks an NPC1L1 gene which encodes or can produce a functional NPC1L1 protein. Included within this embodiment are mutant *npc111* genes comprising a point mutation, truncation or deletion of the genetic coding region or of any regulatory element (e.g., a promoter).

For example, the cell can be isolated from a mutant mouse comprising a homozygous mutation of endogenous, chromosomal NPC1L1 wherein the mouse does not produce any functional NPC1L1 protein (e.g., the mouse described below in Example 22). Moreover, the present invention comprises any cell, tissue, organ, fluid, nucleic acid, peptide or other biological substance derived or isolated from such a mutant mouse, particularly a mutant, transgenic mouse which does not produce any functional NPC1L1, wherein the region of endogenous, chromosomal NPC1L1 deleted, in the mouse, corresponds to nucleotides 790-998 of the nucleotide sequence set forth in SEQ ID NO: 45.

The isolated cell can be isolated or derived, for example, from the duodenum, gall bladder, liver, small intestine or stomach of the mutant mouse. Further, the cell can be an enterocyte.

The *npc111*<sup>-</sup> mutant cells are useful, for example, for use in control experiments in screening assays (see e.g., supra) since they lack any NPC1L1-dependent uptake or binding of sterol, 5 $\alpha$ -stanol or ezetimibe. The level of inhibition caused by a particular sample, in a screening assay, can be compared to that of an assay performed with the mutant cell. Ideally, though by no means necessarily, in a screening assay, for example, as described herein, the same amount of binding will be observed by a non-mutant cell or cell membrane, in the presence of an antagonist, as is observed in connection with a mutant *npc111* cell or cell membrane alone.

## EXAMPLES

The following examples are provided to more clearly describe the present invention and should not be construed to limit the scope of the invention in any way.

### 5 Example 1: Cloning and Expression of Rat, Mouse and Human NPC1L1.

Rat *NPC1L1*, mouse *NPC1L1* or human *NPC1L1* can all conveniently be amplified using polymerase chain reaction (PCR). In this approach, DNA from a rat, mouse or human cDNA library can be amplified using appropriate primers and standard PCR conditions. Design of primers and optimal amplification conditions  
10 constitute standard techniques which are commonly known in the art.

An amplified *NPC1L1* gene may conveniently be expressed, again, using methods which are commonly known in the art. For example, NPC1L1 may be inserted into a pET-based plasmid vector (Stratagene; La Jolla, CA), downstream of the T7 RNA polymerase promoter. The plasmid may then be transformed into a T7  
15 expression system (e.g., BL21DE3 E.coli cells), grown in a liquid culture and induced (e.g., by adding IPTG to the bacterial culture).

### Example 2: Direct Binding Assay.

Membrane preparation: Caco2 cells transfected with an expression vector containing a polynucleotide encoding NPC1L1 (e.g., SEQ ID NO: 2, 4 or 12)  
20 are harvested by incubating in 5 mM EDTA/phosphate-buffered saline followed by repeated pipeting. The cells are centrifuged 5 min at 1000 x g. The EDTA/PBS is decanted and an equal volume of ice-cold 50mM Tris-HCl, pH 7.5 is added and cells are broken up with a Polytron (PT10 tip, setting 5, 30 sec). Nuclei and unbroken cells are sedimented at 1000 x g for 10 min and then the supernatant is centrifuged at  
25 50,000 x g for 10 min. The supernatant is decanted, the pellet is resuspended by Polytron, a sample is taken for protein assay (bicinchoninic acid, Pierce), and the tissue is again centrifuged at 50,000 x g. Pellets are stored frozen at -20°C.

Binding assay: For saturation binding, four concentrations of [<sup>3</sup>H]-ezetimibe (15 Ci/mmol) are incubated without and with 10<sup>-5</sup> M ezetimibe in triplicate  
30 with 50 µg of membrane protein in a total volume of 200 µl of 50 mM Tris-HCl, pH 7.5, for 30 min at 30°C. Samples are filtered on GF/B filters and washed three times with 2 ml of cold Tris buffer. Filters are dried in a microwave oven, impregnated



with Meltilex wax scintillant, and counted at 45% efficiency. For competition binding assays, five concentrations of a sample are incubated in triplicate with 18 nM [<sup>3</sup>H]-ezetimibe and 70 µg of membrane protein under the conditions described above. Curves are fit to the data with Prism (GraphPad Software) nonlinear least-squares  
5 curve-fitting program and K<sub>i</sub> values are derived from IC<sub>50</sub> values according to Cheng and Prusoff (Cheng, Y. C., *et al.*, (1973) *Biochem. Pharmacol.* 22: 3099-3108).

**Example 3A: SPA Assay.**

For each well of a 96 well plate, a reaction mixture of 10 µg human, mouse or rat NPC1L1-CHO overexpressing membranes (Biosignal) and 200 µg/well  
10 YSi-WGA-SPA beads (Amersham) in 100 µl is prepared in NPC1L1 assay buffer (25 mM HEPES, pH 7.8, 2 mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, 125 mM NaCl, 0.1% BSA). A 0.4 nM stock of ligand- [<sup>125</sup>I]-ezetimibe- is prepared in the NPC1L1 assay buffer. The above solutions are added to a 96-well assay plate as follows: 50 µl NPC1L1 assay  
15 buffer, 100 µl of reaction mixture, 50 µl of ligand stock (final ligand concentration is 0.1 nM). The assay plates are shaken for 5 minutes on a plate shaker, then incubated for 8 hours before cpm/well are determined in Microbeta Trilux counter (PerkinElmer).

These assays will indicate that [<sup>125</sup>I]-ezetimibe binds to the cell membranes expressing human, mouse or rat NPC1L1. Similar results will be  
20 obtained if the same experiment is performed with radiolabeled cholesterol (*e.g.*, <sup>125</sup>I-cholesterol).

**Example 3A: Alternate SPA Assay.**

The final concentrations should be: 1 nM <sup>35</sup>S-2 (K<sub>m</sub> ~ 2-5 nM, ~50,000 dpm/assay);  
25 1 µg membranes (~1-2 nM receptor); 0.007%-0.03% taurocholate (0.140 µl 1% stock); 0.01 0%-0.05% digitonin (0.200 µl 1% stock); 5% DMSO (1.00 µl inhibitors).

In each well of a 96 well plate is put 1 µl DMSO inhibitor solution, and then the radioligand and detergents are added as a 2X solution in 10 µl buffer A.  
30 Shake for a minute to be sure the inhibitor and ligand are mixed, before initiating with 9 µl diluted receptor solution in buffer A. After shaking again, the plate is incubated at 37°C for 2 hours. Then WGA beads (0.3 mg) are added as a 3 µl suspension in

buffer A, then shake for 30 minutes. Similar results are obtained if membranes are pre-incubated with beads for 30 minutes before adding ligands. Finally, dilute to 300  $\mu$ l with buffer A, cover the plate, spin at 3,000 rpm x 5 min, and read at 2 minutes per well in the "Microbeta" counter.

## 5 Stocks

Ligand:  $^{35}\text{S}$ -2 is 525.42 nM, 0616  $\mu\text{Ci}/\mu\text{l}$ , in acetonitrile; spec. act. =  $3.8916 \times 10^{-4}$  fmol/dpm; 1168 Ci/mmol)

Membranes: 3<sup>rd</sup> batch recombinant human expressed in HEK-293 cells; 20.2  $\mu\text{g}/\mu\text{l}$  stock; ~20–40 pmol NPC1L1/mg protein

10 Buffer A: 26 mM  $\text{NaHCO}_3$ ; 0.96 mM  $\text{NaH}_2\text{PO}_4$ ; 5 mM HEPES; optional addition of 5.5 mM glucose; 117 mM NaCl; 5.4 mM KCl

### **Example 4: Cholesterol Uptake Assay.**

CHO cells expressing either SR-B1 or three different clones of rat NPC1L1 or one clone of mouse NPC1L1 were starved overnight in cholesterol free media then dosed with  $[^3\text{H}]$ -cholesterol in a mixed synthetic micelle emulsion for 4 min, 8 min, 12 min or 24 min in the absence or presence of 10  $\mu\text{M}$  ezetimibe. The cells were harvested and the lipids were organically extracted. The extracted lipids were spotted on thin-layer chromatography (TLC) plates and resolved within an organic vapor phase. The free cholesterol bands for each assay were isolated and counted in a scintillation counter.

The SR-B1 expressing cells exhibited an increase in  $[^3\text{H}]$ -cholesterol uptake as early as 4 min which was also inhibited by ezetimibe. The three rat clones and the one mouse clone appeared to give background levels of  $[^3\text{H}]$ -cholesterol uptake which was similar to that of the untransformed CHO cell.

25 These experiments will yield data demonstrating that CHO cells can perform mouse, rat and human NPC1L1-dependent uptake of  $[^3\text{H}]$ -cholesterol when more optimal experimental conditions are developed.

### **Example 5: Expression of Rat NPC1L1 in Wistar Rat Tissue.**

30 In these experiments, the expression of rat *NPC1L1* mRNA, in several rat tissues, was evaluated. The tissues evaluated were esophagus, stomach, duodenum, jejunum, ileum, proximal colon, distal colon, liver, pancreas, heart, aorta, spleen, lung, kidney, brain, muscle, testes, ovary, uterus, adrenal gland and

thyroid gland. Total RNA samples were isolated from at least 3 male and 3 female animals and pooled. The samples were then subjected to real time quantitative PCR using Taqman analysis using standard dual-labeled fluorogenic oligonucleotide probes. Typical probe design incorporated a 5' reporter dye (e.g., 6FAM (6-carboxyfluorescein) or VIC) and a 3' quenching dye (e.g., TAMRA (6-carboxytetramethyl-rhodamine)).

rat *NPC1L1*:

Forward: TCTTCACCCTTGCTCTTTGC (SEQ ID NO: 14)

Reverse: AATGATGGAGAGTAGGTTGAGGAT (SEQ ID NO: 15)

Probe: [6FAM]TGCCACCTTTGTTGTCTGCTACC[TAMRA]  
(SEQ ID NO: 16) rat  $\beta$ -actin:

Forward: ATCGCTGACAGGATGCAGAAG (SEQ ID NO: 17)

Reverse: TCAGGAGGAGCAATGATCTTGA (SEQ ID NO: 18)

Probe: [VIC]AGATTACTGCCCTGGCTCCTAGCACCAT[TAMRA]  
(SEQ ID NO: 19)

PCR reactions were run in 96-well format with 25  $\mu$ l reaction mixture in each well containing: Platinum SuperMix (12.5  $\mu$ l), ROX Reference Dye (0.5  $\mu$ l), 50 mM magnesium chloride (2  $\mu$ l), cDNA from RT reaction (0.2  $\mu$ l). Multiplex reactions contained gene specific primers at 200 nM each and FAM labeled probe at 100 nM and gene specific primers at 100 nM each and VIC labeled probe at 50 nM. Reactions were run with a standard 2-step cycling program, 95°C for 15 sec and 60°C for 1 min, for 40 cycles.

The highest levels of expression were observed in the duodenum, jejunum and ileum tissue. These data indicate that NPC1L1 plays a role in cholesterol absorption in the intestine.

**Example 6: Expression of Mouse *NPC1L1* in Mouse Tissue.**

In these experiments, the expression of mouse *NPC1L1* mRNA, in several tissues, was evaluated. The tissues evaluated were adrenal gland, BM, brain, heart, islets of langerhans, LI, small intestine, kidney, liver, lung, MLN, PLN, muscle, ovary, pituitary gland, placenta, Peyers Patch, skin, spleen, stomach, testes, thymus, thyroid gland, uterus and trachea. Total RNA samples were isolate from at least 3 male and 3 female animals and pooled. The samples were then subjected to real time quantitative PCR using Taqman analysis using the following primers and probes:

mouse *NPC1L1*:

Forward: ATCCTCATCCTGGGCTTTGC (SEQ ID NO: 20)

Reverse: GCAAGGTGATCAGGAGGTTGA (SEQ ID NO: 21)

Probe: [6FAM]CCCAGCTTATCCAGATTTTCTTCTTCCGC[TAMRA]

5 (SEQ ID NO: 22)

The highest levels of expression were observed in the Peyer's Patch, small intestine, gall bladder and stomach tissue. These data are consistent with a cholesterol absorption role for NPC1L1 which takes place in the digestive system.

**Example 7: Expression of Human NPC1L1 in Human Tissue.**

10 In these experiments, the expression level of human NPC1L1 mRNA was evaluated in 2045 samples representing 46 normal tissues. Microarray-based gene expression analysis was performed on the Affymetrix HG-U95 GeneChip using a cRNA probe corresponding to base pairs 4192-5117 (SEQ ID NO: 43) in strict accordance to Affymetrix's established protocols. Gene Chips were scanned under

15 low photo multiplier tube (PMT), and data were normalized using either Affymetrix MAS 4.0 or MAS 5.0 algorithms. In addition "spike ins" for most samples were used to construct a standard curve and obtain RNA concentration values according Gene Logic algorithms and procedures. A summary of these results are indicated, below, in Table 2.

Table 2. Expression level of *NPC1L1* mRNA in various human tissues.

Tissue	Present	Absent	Lower 25%	Median	Upper 75%	Tissue	Present	Absent	Lower 25%	Median	Upper 75%
Adipose	2 of 32	30 of 32	-2.45	1.16	11.23	Liver	32 of 34	2 of 34	325.74	427.77	540.1
Adrenal Gland	0 of 12	12 of 12	-23.54	-4.47	10.51	Lung	2 of 93	91 of 93	-3.47	11.03	22.34
Appendix	0 of 3	3 of 3	-9.02	-6.59	38.19	Lymph Node	0 of 11	11 of 11	-1.78	-0.19	1.34
Artery	0 of 3	3 of 3	-6.59	-4.57	9.68	Muscles	0 of 39	39 of 39	-21.57	8.15	26.73
Bladder	1 of 6	5 of 6	-22	-7.95	-1.89	Myometrium	8 of 105	98 of 105	-3.96	4.87	17.55
Bone	0 of 3	3 of 3	-1.84	9.3	19.53	Omentum	0 of 15	15 of 15	-14.25	-1.6	19.58
Breast	4 of 80	76 of 80	-4.07	3.13	14.67	Ovary	1 of 74	73 of 74	0.5	17.51	38.28
Cardiolum	0 of 5	5 of 5	-3.04	3.24	18.38	Pancreas	0 of 34	34 of 34	-87.08	-53.2	-24.14
Cervix	3 of 101	98 of 101	-7.56	-0.07	20.89	Placenta	0 of 5	5 of 5	-20.4	-3.44	18.91
Colon	9 of 151	142 of 151	-10.19	0.31	18.36	Prostate	0 of 32	32 of 32	1.08	15.58	27.24
Cortex Frontal Lobe	0 of 7	7 of 7	1.4	8.66	11.75	Spleen	1 of 43	42 of 43	-9.28	-1.49	9.8
Cortex Temporal Lobe	0 of 3	3 of 3	7.1	8.5	15.87	Right Atrium	4 of 169	165 of 169	-19.32	-6.58	7.72
Duodenum	59 of 61	2 of 61	519.23	817.13	1101.67	Right Ventricle	1 of 160	159 of 160	-24.01	-4.48	18.86
Endometrium	0 of 21	21 of 21	-14.43	-6.38	2.78	Skin	0 of 59	59 of 59	-12.68	1.5	22.77
Esophagus	1 of 27	26 of 27	-10.93	-4.97	12.48	Small Intestine	46 of 63	22 of 63	21.21	493.93	939.2
Fallopian Tube	3 of 51	48 of 51	5.02	13.24	26.77	Soft Tissues	1 of 6	5 of 6	-1.99	2.6	5.32
Gallbladder	0 of 0	0 of 0	205.76	273.39	422.6	Spleen	0 of 31	31 of 31	-8.41	-0.31	9.5
Heart	0 of 3	3 of 3	3.33	11.19	11.66	Stomach	7 of 47	40 of 47	19.02	52.29	117.09
Hippocampus	0 of 5	5 of 5	8.25	9.11	19.83	Testis	0 of 5	5 of 5	-4.51	1.22	11.2
Kidney	4 of 86	82 of 86	-8.38	3.41	16.46	Thymus	1 of 71	70 of 71	-6.26	2.51	11.67
Larynx	0 of 4	4 of 4	-13.75	-0.81	8.54	Thyroid Gland	1 of 18	17 of 18	-12.22	2.84	17.86
Left Atrium	2 of 141	139 of 141	-18.9	-4.58	6.84	Uterus	0 of 58	58 of 58	-10.67	1.59	16.01
Left Ventricle	0 of 15	15 of 15	-21.19	-9.89	17.7	WBC	3 of 40	37 of 40	-16.45	-0.72	25.18

Shaded data corresponds to tissues wherein the highest levels of *NPC1L1* mRNA was detected. The "Present" column indicates the proportion of specified tissue samples evaluated wherein *NPC1L1* mRNA was detected. The "Absent" column indicates the proportion of specified tissue samples evaluated wherein *NPC1L1* RNA was not detected. The "lower 25%", "median" and "upper 75%" columns indicate statistical distribution of the relative *NPC1L1* signal intensities observed for each set of tissue evaluated.

**Example 8: Distribution of Rat *NPC1L1*, Rat *IBAT* or Rat *SR-B1* mRNA in Rat Small Intestine.**

In these experiments, the distribution of rat *NPC1L1* mRNA along the proximal-distal axis of rat small intestines was evaluated. Intestines were isolated from five independent animals and divided into 10 sections of approximately equal length. Total RNA was isolated and analyzed, by real time quantitative PCR using Taqman analysis, for localized expression levels of rat *NPC1L1*, rat *IBAT* (ileal bile acid transporter) or rat *SR-B1* mRNA. The primers and probes used in the analysis were:

rat NPC1L1:

Forward: TCTTCACCCTTGCTCTTTGC (SEQ ID NO: 23)

Reverse: AATGATGGAGAGTAGGTTGAGGAT (SEQ ID NO: 24)

Probe: [6FAM]TGCCACCTTTGTTGTCTGCTACC[TAMRA]

5 (SEQ ID NO: 25) rat Villin:

Forward: AGCACCTGTCCACTGAAGATTTC (SEQ ID NO: 26)

Reverse: TGGACGCTGAGCTTCAGTTCT (SEQ ID NO: 27)

Probe: [VIC]CTTCTCTGCGCTGCCTCGATGGAA[TAMRA] (SEQ

ID NO: 28) rat SR-B1:

10 Forward: AGTAAAAAGGGCTCGCAGGAT (SEQ ID NO: 29)

Reverse: GGCAGCTGGTGACATCAGAGA (SEQ ID NO: 30)

Probe: [6FAM]AGGAGGCCATGCAGGCCTACTCTGA[TAMRA]

(SEQ ID NO: 31) rat IBAT:

Forward: GAGTCCACGGTCAGTCCATGT (SEQ ID NO: 32)

15 Reverse: TTATGAACAACAATGCCAAGCAA (SEQ ID NO: 33)

Probe: [6FAM]AGTCCTTAGGTAGTGGCTTAGTCCCTGGAAGC

TC[TAMRA] (SEQ ID NO: 34)

The mRNA expression levels of each animal intestinal section were analyzed separately, then the observed expression level was normalized to the observed level of villin mRNA in that intestinal section. The observed, normalized mRNA expression levels for each section were then averaged.

The expression level of *NPC1L1* and *SR-B1* were highest in the jejunum (sections 2-5) as compared to that of the more distal ileum sections. Since the jejunum is believed to be the site of cholesterol absorption, these data suggest such a role for rat *NPC1L1*. *IBAT* distribution favoring the ileum is well documented and served as a control for the experiment.

**Example 9: In situ Analysis of Rat NPC1L1 mRNA in Rat Jejunum Tissue.**

The localization of rat *NPC1L1* mRNA was characterized by *in situ* hybridization analysis of rat jejunum serial sections. The probes used in this analysis were:

T7-sense probe: GTAATACGACTCACTATAGGGCCCTGACGGT CCTTCCTGAGGGAATCTTCAC (SEQ ID NO: 35)

**T7-antisense probe:** GTAATACGACTCACTATAGGGCCTGGGAA  
GTTGGTCATGGCCACTCCAGC (SEQ ID NO: 36)

The RNA probes were synthesized using T7 RNA polymerase amplification of a PCR amplified DNA fragment corresponding rat *NPC1L1* nucleotides 3318 to 3672 (SEQ ID NO 1). Sense and anti-sense digoxigenin-UTP labeled cRNA probes were generated from the T7 promoter using the DIG RNA Labeling Kit following the manufacturer's instructions. Serial cryosections rat jejunum were hybridized with the sense and antisense probes. Digoxigenin labeling was detected with the DIG Nucleic Acid Detection Kit based on previous methods. A positive signal is characterized by the deposition of a red reaction product at the site of hybridization.

The anti-sense probe showed strong staining of epithelium along the crypt-villus axis under low magnification (40X). The observed rat *NPC1L1* mRNA expression levels may have been somewhat greater in the crypts than in the villus tips. Under high magnification (200X), staining was observed in the enterocytes but not in the goblet cells. A lack of staining observed with the sense probe (control) confirmed the high specificity of the *NPC1L1* anti-sense signal. These data provided further evidence of the role of rat *NPC1L1* in intestinal cholesterol absorption.

**Example 10: FACS Analysis of Fluorescently Labeled  
Ezetimibe Binding to Transiently Transfected CHO Cells.**

In these experiments, the ability of BODIPY-labeled ezetimibe (Altmann, *et al.*, (2002) Biochim. Biophys. Acta 1580(1): 77-93) to bind to *NPC1L1* and *SR-B1* was evaluated. "BODIPY" is a fluorescent group which was used to detect the BODIPY-ezetimibe. Chinese hamster ovary (CHO) cells were transiently transfected with rat *NPC1L1* DNA (rNPC1L1/CHO), mouse *NPC1L1* DNA (mNPC1L1/CHO), mouse *SR-B1* DNA (mSRBI/CHO) or *EGFP* DNA (EGFP/CHO). EGFP is enhanced green fluorescent protein which was used as a positive control. The transfected CHO cells or untransfected CHO cells were then stained with 100 nM BODIPY-labeled ezetimibe and analyzed by FACS. Control experiments were also performed wherein the cells were not labeled with the BODIPY-ezetimibe and wherein untransfected CHO cells were labeled with the BODIPY-ezetimibe.

No staining was observed in the untransfected CHO, rNPC1L1/CHO or mNPC1L1/CHO cells. Fluorescence was detected in the positive-control

EGFP/CHO cells. Staining was also detected in the mouse SR-B1/CHO cells. These data show that, under the conditions tested, BODIPY-ezetimibe is capable of binding to SR-B1 and that such binding is not ablated by the presence of the fluorescent BODIPY group. When more optimal conditions are determined, BODIPY-ezetimibe will be shown to label the rNPC1L1/CHO and mNPC1L1/CHO cells.

**Example 11: FACS Analysis of Transiently Transfected CHO Cells Labeled with Anti-FLAG Antibody M2.**

In these experiments, the expression of FLAG-tagged NPC1L1 on CHO cells was evaluated. CHO cells were transiently transfected with mouse *NPC1L1* DNA, rat *NPC1L1* DNA, FLAG- rat *NPC1L1* DNA or FLAG- mouse *NPC1L1* DNA. The 8 amino acid FLAG tag used was DYKDDDDK (SEQ ID NO: 37) which was inserted on the amino-terminal extracellular loop just past the secretion signal sequence. The cells were incubated with commercially available anti-FLAG monoclonal mouse antibody M2 followed by a BODIPY-tagged anti-mouse secondary antibody. The treated cells were then analyzed by FACS.

The M2 antibody stained the CHO cells transfected with FLAG-rat *NPC1L1* DNA and with FLAG-mouse NPC1L1. No staining was observed in the CHO cells transfected with mouse *NPC1L1* DNA and with rat *NPC1L1* DNA. These data showed that rat NPC1L1 and mouse NPC1L1 possess no significant, inherent fluorescence and are not bound by the anti-FLAG antibody. The observed, FLAG-dependent labeling of the cells indicated that the FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 proteins are localized at the cell membrane of the CHO cells.

**Example 12: FACS Analysis of FLAG-rat NPC1L1-EGFP Chimera in Transiently Transfected CHO Cells.**

In these experiments, the surface and cytoplasmic localization of rat NPC1L1 in CHO cells was evaluated. CHO cells were transiently transfected with FLAG- rat *NPC1L1* DNA or with FLAG-rat *NPC1L1-EGFP* DNA. In these fusions, the FLAG tag is at amino-terminus of rat NPC1L1 and EGFP fusion is at the carboxy-terminus of rat NPC1L1. The cells were then stained with the M2 anti-FLAG mouse (primary) antibody followed by secondary staining with a BODIPY-labeled anti-mouse antibody. In control experiments, cells were stained with only the secondary antibody and not with the primary antibody (M2). The stained cells were then analyzed by FACS.



In a control experiment, FLAG-rat NPC1L1 transfected cells were stained with BODIPY anti-mouse secondary antibody but not with the primary antibody. The data demonstrated that the secondary, anti-mouse antibody possessed no significant specificity for FLAG-rat NPC1L1 and that the FLAG-rat NPC1L1, itself, possesses no significant fluorescence.

In another control experiment, unlabeled FLAG-rat NPC1L1-EGFP cells were FACS analyzed. In these experiments, autofluorescence of the enhanced green fluorescent protein (EGFP) was detected.

FLAG-rat NPC1L1 cells were stained with anti-FLAG mouse antibody M2 and with the BODIPY-labeled anti-mouse secondary antibody and FACS analyzed. The data from this analysis showed that the cells were labeled with the secondary, BODIPY-labeled antibody which indicated expression of the FLAG-rat NPC1L1 protein on the surface of the CHO cells.

FLAG-rat NPC1L1-EGFP cells were stained with anti-FLAG mouse antibody M2 and with the BODIPY-labeled anti-mouse secondary antibody and FACS analyzed. The data from this analysis showed that both markers (BODIPY and EGFP) were present indicating surface expression of the chimeric protein. The data also indicated that a portion of the protein was located within the cells and may be associated with transport vesicles. These data supported a role for rat NPC1L1 in vesicular transport of cholesterol or protein expressed in subcellular organelles such as the rough endoplasmic reticulum.

**Example 13: FACS Analysis and Fluorescent Microscopy of FLAG-rat NPC1L1-EGFP Chimera in a Cloned CHO Cell Line.**

In these experiments, the cellular localization of rat NPC1L1 was evaluated by FACS analysis and by immunohistochemistry. CHO cells were transfected with FLAG-rat *NPC1L1-EGFP* DNA and stained with anti-FLAG mouse antibody M2 and then with a BODIPY-labeled anti-mouse secondary antibody. In the fusion, the FLAG tag is at the amino-terminus of rat NPC1L1 and the enhanced green fluorescent protein (EGFP) tag is located at the carboxy-terminus of the rat NPC1L1. The stained cells were then analyzed by FACS and by fluorescence microscopy.

Cells transfected with FLAG-rat *NPC1L1-EGFP* DNA were stained with the anti-FLAG mouse antibody M2 and then with the BODIPY-labeled anti-

mouse secondary antibody. FACS analysis of the cells detected both markers indicating surface expression of the chimeric protein.

FLAG-rat NPC1L1-EGFP transfected cells were analyzed by fluorescent microscopy at 63X magnification. Fluorescent microscopic analysis of the cells indicated non-nuclear staining with significant perinuclear organelle staining. Resolution of the image could not confirm the presence of vesicular associated protein. These data indicated that the fusion protein was expressed on the cell membrane of CHO cells.

**Example 14: Generation of Polyclonal Anti-rat NPC1L1 Rabbit Antibodies.**

Synthetic peptides (SEQ ID NO: 39-42) containing an amino- or carboxy-terminal cysteine residue were coupled to keyhole limpet hemocyanin (KLH) carrier protein through a disulfide linkage and used as antigen to raise polyclonal antiserum in New Zealand white rabbits (range 3-9 months in age). The KLH-peptide was emulsified by mixing with an equal volume of Freund's Adjuvant, and injected into three subcutaneous dorsal sites. Prior to the 16 week immunization schedule a pre-immune sera sample was collected which was followed by a primary injection of 0.25 mg KLH-peptide and 3 scheduled booster injections of 0.1 mg KLH-peptide. Animals were bled from the auricular artery and the blood was allowed to clot and the serum was then collected by centrifugation

The anti-peptide antibody titer was determined with an enzyme linked immunosorbent assay (ELISA) with free peptide bound in solid phase (1µg/well). Results are expressed as the reciprocal of the serum dilution that resulted in an OD<sub>450</sub> of 0.2. Detection was obtained using the biotinylated anti-rabbit IgG, horse radish peroxidase-streptavidin (HRP-SA) conjugate, and ABTS.

**Example 15: FACS Analysis of Rat NPC1L1 Expression in CHO Cells Transiently Transfected with Rat NPC1L1 DNA Using Rabbit Anti-rat NPC1L1 Antisera.**

In these experiments, the expression of rat NPC1L1 on the surface of CHO cells was evaluated. CHO cells were transfected with rat *NPC1L1* DNA, then incubated with either rabbit preimmune serum or with 10 week anti-rat NPC1L1 serum described, above, in Example 14 (i.e., A0715, A0716, A0867 or A0868). Cells labeled with primary antisera were then stained with a BODIPY-modified anti-rabbit secondary antibody followed by FACS analysis.

No antibody surface labeling was observed for any of the pre-immune sera samples. Specific cell surface labeling of rat NPC1L1 transfected cells was observed for both A0715 and A0868. Antisera A0716 and A0867 did not recognize rat NPC1L1 surface expression in this assay format. This indicates that the native, unfused rat NPC1L1 protein is expressed in the CHO cells and localized to the CHO cell membranes. Cell surface expression of NPC1L1 is consistent with a role in intestinal cholesterol absorption.

**Example 16: FACS Analysis of CHO Cells Transiently Transfected with FLAG-Mouse NPC1L1 DNA or FLAG-rat NPC1L1 DNA or Untransfected CHO Cells Using Rabbit Anti-rat NPC1L1 Antisera.**

In these experiments, the expression of FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 in CHO cells was evaluated. CHO cells were transiently transfected with FLAG-mouse *NPC1L1* DNA or with FLAG-rat *NPC1L1* DNA. The FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 transfected cells were labeled with either A0801, A0802, A0715 or A0868 sera (see Example 14) or with anti-FLAG antibody, M2. The labeled cells were then stained with BODIPY-labeled anti-rabbit secondary antibody and FACS analyzed. The untransfected CHO cells were analyzed in the same manner as the transfected cell lines.

Positive staining of the untransfected CHO cells was not observed for any of the antisera tested. Serum A0801-dependent labeling of FLAG-rat NPC1L1 transfected cells was observed but such labeling of FLAG-mouse NPC1L1 transfected cells was not observed. Serum A0802-dependent labeling of FLAG-mouse NPC1L1 or FLAG-rat NPC1L1 transfected cells was not observed. Strong serum A0715-dependent labeling of FLAG-rat NPC1L1 transfected cells was observed and weak serum A0715-dependent labeling of FLAG-mouse NPC1L1 transfected cells was observed. Weak serum A0868-dependent labeling of rat NPC1L1 and mouse NPC1L1 transfected cells was observed. Strong Anti-FLAG M2 antibody-dependent labeling of FLAG-rat NPC1L1 and FLAG-mouse NPC1L1 transfected cells was observed. The strong M2 staining is likely to be due to the fact that M2 is an affinity-purified, monoclonal antibody of known concentration. In contrast, the respective antisera are polyclonal, unpurified and contain an uncertain concentration of anti-rat NPC1L1 antibody. These data provide further evidence that the FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 proteins are expressed in CHO cells and localized to

the CHO cell membranes. Cell surface expression of NPC1L1 is consistent with a role in intestinal cholesterol absorption.

**Example 17: Immunohistochemical Analysis of Rat Jejunum Tissue with Rabbit Anti-rat NPC1L1 Antisera A0715.**

5                   In these experiments, the localization of rat NPC1L1 in rat jejunum was analyzed by immunohistochemistry. Rat jejunum was removed, immediately embedded in O.C.T. compound and frozen in liquid nitrogen. Sections (6µm) were cut with a cryostat microtome and mounted on glass slides. Sections were air dried at room temperature and then fixed in Bouin's fixative. Streptavidin-biotin-peroxidase  
10 immunostaining was carried out using Histostain-SP kit. Endogenous tissue peroxidase activity was blocked with a 10 minute incubation in 3% H<sub>2</sub>O<sub>2</sub> in methanol, and nonspecific antibody binding was minimized by a 45 minute incubation in 10% nonimmune rabbit serum. Sections were incubated with a rabbit anti-rat NPC1L1 antisera A0715 or A0868 at a 1: 500 dilution at 4°C, followed by incubation with  
15 biotinylated goat anti-rabbit IgG and with streptavidin-peroxidase. Subsequently, the sections were developed in an aminoethyl carbazole (AEC)-H<sub>2</sub>O<sub>2</sub> staining system and counterstained with hematoxylin and examined by microscopy. A positive reaction using this protocol is characterized by the deposition of a red reaction product at the site of the antigen-antibody reaction. Nuclei appeared blue from the hematoxylin  
20 counterstain. Controls were performed simultaneously on the neighboring sections from the same tissue block. Control procedures consisted of the following: (1) substitute the primary antibody with the pre-immune serum, (2) substitute the primary antibody with the non-immune rabbit serum, (3) substitute the primary antibody with PBS, (4) substitute the second antibody with PBS.

25                   The example shows tissue stained with anti-rat NPC1L1 sera A0715 or with the preimmune sera analyzed at low magnification (40X) and at high magnification (200X). The A0715-stained tissue, at low magnification, showed positive, strong staining of the villi epithelial layer (enterocytes). The A0715-stained tissue at high magnification showed positive, strong staining of the enterocyte apical  
30 membranes. No staining was observed in tissue treated only with preimmune sera. Similar results were obtained with sera A0868. These data indicate that rat NPC1L1 is expressed in rat jejunum which is consistent with a role in intestinal cholesterol absorption.

**Example 18: Labeled Cholesterol Uptake Assay.**

In this example, the ability of CHO cells stably transfected with rat NPC1L1 to take up labeled cholesterol was evaluated. In these assays, cholesterol uptake, at a single concentration, was evaluated in a pulse-chase experiment. The data generated in these experiments are set forth, below, in Table 3.

**Cells:**

- A. CHO cells stably transfected with rat *NPC1L1* cDNA
- B. CHO background (no transfection)

Cells were seeded at 500,000 cells/ well (mL) in 12-well plates.

**10 Procedure:**

All reagents and culture plates were maintained at 37°C unless otherwise noted.

**Starve.** The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed and the cells were rinsed with serum-free HAMS media. The serum-free media was then replaced with 1 mL "starve" media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS)).

One plate of each cell line was starved overnight. The remaining 2 plates were designated "No Starve" (see below).

**Pre-Incubation.** Media was removed from all plates, rinsed with serum-free HAMS and replaced with starve media for 30 minutes.

**<sup>3</sup>H-Cholesterol Pulse.** The following was added directly to each well.

0.5μCi <sup>3</sup>H-cholesterol (~1.1 X 10<sup>6</sup> dpm/well) in 50μl of a mixed bile salt micelle.

4.8 mM sodium taurocholate (2.581 mg/mL)

0.6 mM sodium oleate (0.183 mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in "starve" media by ultrasonic vibration

Final media cholesterol concentration = 5μg/mL

Labeled cholesterol pulse time points were 0, 4, 12 and 24 minutes.

30 Triplicate wells for each treatment were prepared.

**Wash.** At the designated times, media was aspirated and the cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% BSA, pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

**Processing/Analysis.** Cells were digested overnight with 0.2N NaOH, 2mL/well at room temperature. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting. Two additional 50µl aliquots from all wells are assayed for total protein by the Pierce micro BCA method.

- 5 The quantity of labeled cholesterol observed in the cells was normalized by the quantity of protein in the cells.

**Table 3. Uptake of  $^3\text{H}$ -cholesterol by CHO cells transfected with rat *NPC1L1* or mouse *SR-B1* or untransfected CHO cells.**

Time, min	Total Cholesterol, dpm protein $\pm$ sem					Total Cholesterol, dpm/mg protein $\pm$ sem			
	NPC1L1		CHO			NPC1L1		CHO	
After $^3\text{H}$ -Cholesterol					No Starve				
0	2067	$\pm 46$	4568	$\pm 1937$		10754	$\pm 166$	22881	$\pm 9230$
4	2619	$\pm 130$	2868	$\pm 193$		15366	$\pm 938$	15636	$\pm 1471$
12	2868	$\pm 193$	4459	$\pm 170$		15636	$\pm 1471$	24622	$\pm 966$
24	7010	$\pm 89$	7204	$\pm 173$		41129	$\pm 685$	39361	$\pm 1207$
					Starve				
0	1937	$\pm 273$	2440	$\pm 299$		10909	$\pm 1847$	12429	$\pm 1673$
4	3023	$\pm 308$	2759	$\pm 105$		17278	$\pm 1650$	14307	$\pm 781$
12	2759	$\pm 105$	4857	$\pm 186$		14307	$\pm 781$	26270	$\pm 1473$
24	6966	$\pm 72$	7344	$\pm 65$		39196	$\pm 174$	38381	$\pm 161$

dpm=disintegrations per minute  
sem=standard error of the mean

10

#### Example 19: Effect of Ezetimibe on Cholesterol Uptake.

The effect of ezetimibe on the ability of CHO cells stably transfected with mouse or rat *NPC1L1* or mouse *SR-B1* to take up  $^3\text{H}$ -labeled cholesterol was evaluated in pulse-chase experiments. One cDNA clone of mouse *NPC1L1* (C7) and three clones of rat *NPC1L1* (C7, C17 and C21) were evaluated. The ability of CHO

15

cells stably transfected with mouse *SR-BI*, mouse *NPC1L1* and rat *NPC1L1* to take up labeled cholesterol, in the absence of ezetimibe, was also evaluated in the pulse-chase experiments. Data generated in these experiments are set forth, below, in Tables 4 and 5. Additionally, the quantity of total cholesterol taken up by transfected and untransfected CHO cells in the presence of four different unlabeled cholesterol concentrations was also evaluated. The data from these experiments is set forth, below, in Table 6.

**Cells:**

- A. CHO cells stably transfected with rat or mouse *NPC1L1* cDNA
  - B. CHO background (no transfection)
  - C. *SR-BI* transfected CHO cells
- Cells seeded at 500,000 cells / well (mL) in 12-well plates.

**Procedure:**

All reagents and culture plates were maintained at 37°C unless otherwise noted.

**Starve.** The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed and the cells were rinsed with serum-free HAMS media. The serum-free media was then replaced with 1 mL "starve" media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS)). The cells were then starved overnight.

**Pre-Incubation/ pre-dose.** Media was removed from all plates and replaced with fresh starve media and preincubated for 30 minutes. Half of the wells received media containing ezetimibe (stock soln in EtOH; final conc. = 10µM).

**<sup>3</sup>H-Cholesterol Pulse.** The following was added directly to each well:

0.5µCi <sup>3</sup>H-cholesterol (~1.1 X 10<sup>6</sup> dpm/well) in 50µl of a mixed bile salt micelle

4.8mM sodium taurocholate (2.581mg/mL)

0.6 mM sodium oleate (0.183 mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in "starve" media by ultrasonic vibration

Final media cholesterol concentration = 5µg/mL

Labeled cholesterol pulse time points were 4, 12, 24 minutes and 4 hours. Triplicate wells were prepared for each treatment.

**Wash.** At designated times, media was aspirated and cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% bovine serum albumin (BSA), pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

5                   **Processing/Analysis.**

A.     4, 12, 24 minute time points: Cells were digested overnight with 0.2N NaOH, 2mL/well, room temperature. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting.

B.     4 hour time point: The digested cells were analyzed by thin-  
10 layer chromatography to determine the content of cholesterol ester in the cells.

Extracts were spotted onto TLC plates and run for 30 minutes in 2 ml hexane: isopropanol (3: 2) mobile phase for 30 minutes, followed by a second run in 1 ml hexane: isopropanol (3: 2) mobile phase for 15 minutes.

C.     Protein determination of cell extracts. Plates containing a  
15 sample of the cell extracts were placed on orbital shaker at 120 rpm for indicated times and then extracts are pooled into 12 X 75 tubes. Plates were dried and NaOH (2ml/well) added. The protein content of the samples were then determined. Two additional 50µl aliquots from all wells were assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was  
20 normalized to the quantity of protein in the cells.



**Table 4. Total Cholesterol in Transfected CHO Cells in the Presence and Absence of Ezetimibe.**

Clones:	Total Cholesterol, dpm $\pm$ sem				Total Cholesterol, dpm/mg protein $\pm$ sem			
	Vehicle		EZ (10 $\mu$ M)		Vehicle		EZ (10 $\mu$ M)	
	4 Min Pulse							
CHO Control	3413	$\pm$ 417	3222	$\pm$ 26	33443	$\pm$ 4070	31881	$\pm$ 483
SR-BI	14207	$\pm$ 51	10968	$\pm$ 821	118242	$\pm$ 1261	92474	$\pm$ 2902
mNPCIL1(C7)	4043	$\pm$ 419	4569	$\pm$ 222	30169	$\pm$ 3242	30916	$\pm$ 1137
rNPCIL1(C21)	3283	$\pm$ 288	3769	$\pm$ 147	23728	$\pm$ 2111	27098	$\pm$ 689
rNPCIL1(C17)	3188	$\pm$ 232	3676	$\pm$ 134	24000	$\pm$ 832	28675	$\pm$ 527
rNPCIL1(C7)	1825	$\pm$ 806	3268	$\pm$ 121	15069	$\pm$ 6794	27285	$\pm$ 968
	12 Min Pulse							
CHO Control	4710	$\pm$ 246	4532	$\pm$ 165	44208	$\pm$ 2702	43391	$\pm$ 1197
SR-BI	16970	$\pm$ 763	12349	$\pm$ 298	140105	$\pm$ 6523	98956	$\pm$ 4447
mNPCIL1(C7)	6316	$\pm$ 85	6120	$\pm$ 755	45133	$\pm$ 342	41712	$\pm$ 4054
rNPCIL1(C21)	5340	$\pm$ 12	4703	$\pm$ 231	40018	$\pm$ 1181	33985	$\pm$ 1928
rNPCIL1(C17)	4831	$\pm$ 431	4579	$\pm$ 257	37378	$\pm$ 3461	34063	$\pm$ 1619
rNPCIL1(C7)	4726	$\pm$ 272	4664	$\pm$ 63	39100	$\pm$ 2350	38581	$\pm$ 784
	24 Min Pulse							
CHO Control	7367	$\pm$ 232	6678	$\pm$ 215	65843	$\pm$ 1281	61764	$\pm$ 2131
SR-BI	39166	$\pm$ 2152	23558	$\pm$ 1310	324126	$\pm$ 11848	198725	$\pm$ 1713
mNPCIL1(C7)	10616	$\pm$ 121	9749	$\pm$ 482	77222	$\pm$ 1040	74041	$\pm$ 3670
rNPCIL1(C21)	9940	$\pm$ 587	8760	$\pm$ 293	76356	$\pm$ 9618	66165	$\pm$ 2181
rNPCIL1(C17)	8728	$\pm$ 721	8192	$\pm$ 237	70509	$\pm$ 5189	62279	$\pm$ 4352
rNPCIL1(C7)	8537	$\pm$ 148	7829	$\pm$ 204	72134	$\pm$ 1305	63482	$\pm$ 368

EZ = ezetimibe

Table 5. Cholesterol Ester in CHO cells in the Presence or Absence of Ezetimibe.

Clones	Cholesteryl Ester, dpm $\pm$ sem				Cholesteryl Ester, dpm/mg protein $\pm$ sem			
	Vehicle		EZ (10 $\mu$ M)		Vehicle		EZ (10 $\mu$ M)	
	4 Hour Pulse							
CHO Control	652	$\pm$ 13	208	$\pm$ 9	5647	$\pm$ 55	1902	$\pm$ 87
SR-BI	47608	$\pm$ 1292	9305	$\pm$ 401	391067	$\pm$ 14391	72782	$\pm$ 3181
mNPCIL1(C7)	732	$\pm$ 127	453	$\pm$ 118	4994	$\pm$ 827	3057	$\pm$ 776
rNPCIL1(C21)	2667	$\pm$ 90	454	$\pm$ 33	18655	$\pm$ 1032	3193	$\pm$ 265
rNPCIL1(C17)	751	$\pm$ 74	202	$\pm$ 10	5379	$\pm$ 481	1510	$\pm$ 62
rNPCIL1(C7)	462	$\pm$ 25	191	$\pm$ 54	3597	$\pm$ 193	1496	$\pm$ 403

	Free Cholesterol, dpm $\pm$ sem				Free Cholesterol, dpm/mg protein $\pm$ sem			
	Vehicle		EZ (10 $\mu$ M)		Vehicle		EZ (10 $\mu$ M)	
	4 Hour Pulse							
CHO Control	61612	$\pm$ 1227	56792	$\pm$ 568	533876	$\pm$ 17770	519607	$\pm$ 16203
SR-BI	214678	$\pm$ 4241	194519	$\pm$ 474	1762873	$\pm$ 46607	1521341	$\pm$ 4185
mNPCIL1(C7)	79628	$\pm$ 793	77516	$\pm$ 1910	544661	$\pm$ 1269	523803	$\pm$ 10386
rNPCIL1(C21)	71352	$\pm$ 1343	69106	$\pm$ 711	498016	$\pm$ 8171	485460	$\pm$ 4410
rNPCIL1(C17)	78956	$\pm$ 3782	71646	$\pm$ 446	566456	$\pm$ 29204	536651	$\pm$ 7146
rNPCIL1(C7)	75348	$\pm$ 2093	70628	$\pm$ 212	586127	$\pm$ 13932	556855	$\pm$ 7481

EZ =ezetimibe

**Table 6. Uptake of labeled cholesterol in the presence of increasing amounts of unlabeled cholesterol.**

Cold Cholesterol	Total Cholesterol, dpm $\pm$ sem				24 Min Pulse	Total Cholesterol, dpm/mg protein $\pm$ sem			
	CHO Control	SR-B1	mNPC1L1(C7)	rNPC1L1(C21)		CHO Control	SR-B1	mNPC1L1(C7)	rNPC1L1(C21)
3 $\mu$ g/mL	12271 $\pm$ 430	49603 $\pm$ 2428	14250 $\pm$ 1628	10636 $\pm$ 1223	108956 $\pm$ 4513	541562 $\pm$ 13783	140764 $\pm$ 14433	94945 $\pm$ 12916	
10 $\mu$ g/mL	16282 $\pm$ 2438	79967 $\pm$ 8151	25465 $\pm$ 3037	13225 $\pm$ 4336	151283 $\pm$ 23343	880224 $\pm$ 82254	250985 $\pm$ 27481	123433 $\pm$ 34092	
30 $\mu$ g/mL	14758 $\pm$ 1607	71925 $\pm$ 3863	19001 $\pm$ 1530	13218 $\pm$ 1149	135109 $\pm$ 12106	796236 $\pm$ 18952	180435 $\pm$ 12112	111522 $\pm$ 6941	
100 $\mu$ g/mL	16458 $\pm$ 1614	58185 $\pm$ 4548	15973 $\pm$ 1665	11560 $\pm$ 1132	149559 $\pm$ 17977	630143 $\pm$ 3718	147717 $\pm$ 8261	101328 $\pm$ 7191	
	Cholesteryl Ester, dpm $\pm$ sem				4 Hour Pulse	Cholesteryl Ester, dpm/mg protein $\pm$ sem			
	CHO Control	SR-B1	mNPC1L1(C7)	rNPC1L1(C21)		CHO Control	SR-B1	mNPC1L1(C7)	rNPC1L1(C21)
3 $\mu$ g/mL	2737 $\pm$ 114	39596 $\pm$ 1241	1561 $\pm$ 1	4015 $\pm$ 47	22050 $\pm$ 978	382641 $\pm$ 5935	13684 $\pm$ 217	32020 $\pm$ 641	
10 $\mu$ g/mL	1646 $\pm$ 76	17292 $\pm$ 362	998 $\pm$ 36	1866 $\pm$ 33	13323 $\pm$ 606	157914 $\pm$ 3400	8917 $\pm$ 467	14849 $\pm$ 127	
30 $\mu$ g/mL	970 $\pm$ 45	6642 $\pm$ 153	537 $\pm$ 82	970 $\pm$ 49	7627 $\pm$ 325	63547 $\pm$ 1760	4885 $\pm$ 748	7741 $\pm$ 100	
100 $\mu$ g/mL	895 $\pm$ 155	4777 $\pm$ 27	405 $\pm$ 7	777 $\pm$ 16	7133 $\pm$ 1250	45088 $\pm$ 1528	3663 $\pm$ 48	6005 $\pm$ 198	
	Free Cholesterol, dpm $\pm$ sem				4 Hour Pulse	Free Cholesterol, dpm/mg protein $\pm$ sem			
	CHO Control	SR-B1	mNPC1L1(C7)	rNPC1L1(C21)		CHO Control	SR-B1	mNPC1L1(C7)	rNPC1L1(C21)
3 $\mu$ g/mL	89013 $\pm$ 3724	211783 $\pm$ 3268	104343 $\pm$ 2112	92244 $\pm$ 987	717308 $\pm$ 34130	2047695 $\pm$ 16213	914107 $\pm$ 5869	735498 $\pm$ 11209	
10 $\mu$ g/mL	136396 $\pm$ 8566	278216 $\pm$ 10901	196173 $\pm$ 4721	125144 $\pm$ 877	1105118 $\pm$ 76074	2540130 $\pm$ 92471	1753072 $\pm$ 86378	996824 $\pm$ 27830	
30 $\mu$ g/mL	131745 $\pm$ 2922	224429 $\pm$ 2336	149172 $\pm$ 19689	117143 $\pm$ 4976	1036195 $\pm$ 21142	2149315 $\pm$ 78068	1357136 $\pm$ 180264	934772 $\pm$ 43202	
100 $\mu$ g/mL	79336 $\pm$ 4011	231470 $\pm$ 4221	114599 $\pm$ 2803	93538 $\pm$ 1588	632965 $\pm$ 29754	2182022 $\pm$ 436793	1035979 $\pm$ 40329	723225 $\pm$ 21694	
	Cholesteryl Ester, dpm $\pm$ sem				24 Hour Pulse	Cholesteryl Ester, dpm/mg protein $\pm$ sem			
	CHO Control	SR-B1	mNPC1L1(C7)	rNPC1L1(C21)		CHO Control	SR-B1	mNPC1L1(C7)	rNPC1L1(C21)
3 $\mu$ g/mL	37373 $\pm$ 2704	162396 $\pm$ 1644	22986 $\pm$ 940	59377 $\pm$ 953	357629 $\pm$ 14639	1248900 $\pm$ 18565	160328 $\pm$ 6565	401315 $\pm$ 2357	
10 $\mu$ g/mL	33730 $\pm$ 1296	112815 $\pm$ 373	14836 $\pm$ 152	31797 $\pm$ 525	215004 $\pm$ 5942	830231 $\pm$ 12764	98594 $\pm$ 2203	200451 $\pm$ 5229	
30 $\mu$ g/mL	19193 $\pm$ 100	58608 $\pm$ 1413	8878 $\pm$ 355	18963 $\pm$ 380	122071 $\pm$ 1271	446581 $\pm$ 3472	59091 $\pm$ 4267	119728 $\pm$ 2131	
100 $\mu$ g/mL	16761 $\pm$ 198	31280 $\pm$ 1270	8784 $\pm$ 946	14933 $\pm$ 311	103235 $\pm$ 1739	272796 $\pm$ 13392	60670 $\pm$ 4597	96215 $\pm$ 1023	
	Free Cholesterol, dpm $\pm$ sem				24 Hour Pulse	Free Cholesterol, dpm/mg protein $\pm$ sem			
	CHO Control	SR-B1	mNPC1L1(C7)	rNPC1L1(C21)		CHO Control	SR-B1	mNPC1L1(C7)	rNPC1L1(C21)
3 $\mu$ g/mL	248985 $\pm$ 4207	357819 $\pm$ 4519	285610 $\pm$ 5187	227244 $\pm$ 1016	1352637 $\pm$ 18984	2752957 $\pm$ 24984	1993356 $\pm$ 46968	1536023 $\pm$ 10304	
10 $\mu$ g/mL	231208 $\pm$ 8927	269822 $\pm$ 3872	311777 $\pm$ 8227	231666 $\pm$ 1618	1477414 $\pm$ 28584	1984473 $\pm$ 18420	2069380 $\pm$ 25517	1461157 $\pm$ 38517	
30 $\mu$ g/mL	203566 $\pm$ 6008	225373 $\pm$ 3932	279604 $\pm$ 6612	209372 $\pm$ 3386	1294878 $\pm$ 18119	1716066 $\pm$ 52381	1859476 $\pm$ 42947	1321730 $\pm$ 3452	
100 $\mu$ g/mL	178424 $\pm$ 2379	167082 $\pm$ 2211	229832 $\pm$ 4199	182678 $\pm$ 7709	1099648 $\pm$ 25160	1455799 $\pm$ 9883	1599244 $\pm$ 76938	1177546 $\pm$ 31191	

**Example 20: Labeled Cholesterol Uptake Assay.**

5

In this example, the ability of CHO cells transiently transfected with rat *NPC1L1* or mouse *SR-B1* to take up labeled cholesterol was evaluated. Also evaluated was the ability of rat *NPC1L1* to potentiate the ability of CHO cells transfected with mouse *SR-B1* to take up labeled cholesterol. In these assays,

cholesterol uptake, at a single concentration, was evaluated in pulse-chase experiments. The data generated in these experiments are set forth, below, in Table 7.

Cells:

- 5           A.     CHO background cells (mock transfection).  
          B.     CHO cells transiently transfected with mouse SR-B1.  
          C.     CHO transiently transfected with rat *NPC1L1* cDNAs  
                  (n=8 clones).

Transiently transfected cells were seeded at 300,000 cells / well (mL)  
in 12-well plates.

10   **Procedure:**

All reagents and culture plates were maintained at 37°C unless  
otherwise noted.

- Starve.** The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS)  
was removed from the cells and replaced with 1 mL "starve" media (F12 HAMS,  
15   Pen/Strep, 5% lipoprotein deficient serum (LPDS). Cells were starved for 1 hour.

**<sup>3</sup>H-Cholesterol Pulse.** The following was added directly to each well.

- 0.5μCi <sup>3</sup>H-cholesterol (~1.1 X 10<sup>6</sup> dpm/well) in 50μl of a  
mixed bile salt micelle.  
                  4.8mM sodium taurocholate (2.581mg/mL)  
20               0.6 mM sodium oleate (0.183 mg/mL)  
                  0.25 mM cholesterol (0.1 mg/mL)  
                  Dispersed in "starve" media by ultrasonic vibration  
                  Final media cholesterol concentration = 5μg/mL

Labeled cholesterol pulse time points were 24 Min and 4 hours.

- 25   Triplicate wells for each treatment.

**Wash.** At the designated times, media was aspirated and cells were  
washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% BSA, pH 7.4) and  
once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

**Processing/Analysis.**

- 30           A.     24 minute time point: Cells were digested overnight with  
0.2N NaOH, 2mL/well at room temp. One 1.5 mL aliquot was removed from each  
well, neutralized & counted for radioactivity by scintillation counting.

B. 4 hour time point: The digested cells were analyzed by thin-layer chromatography to determine the content of cholesterol ester in the cells.

The extracts were spotted onto thin layer chromatography plates and run in 2 ml hexane: isopropanol (3: 2) containing mobile phase for 30 minutes,  
5 followed by a second run in 1 ml hexane: isopropanol (3: 2) containing mobile phase for 15 min.

C. Protein determination of cell extracts: Plates containing a sample of the cell extracts were placed on orbital shaker at 120 rpm for indicated times and then extracts are pooled into 12X75 tubes. Plates were dried and NaOH  
10 (2ml/well) added. The protein content of the samples were then determined. Two additional 50µl aliquots from all wells were assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized to the quantity of protein in the cells.

Table 7. Labeled cholesterol uptake in transiently transfected CHO cells.

Transfection	Total Cholesterol, $\pm$ sem	
	dpm	dpm/mg protein
	24 Min Pulse	
CHO Control (mock)	4721 $\pm$ 436	49024 $\pm$ 4328
SR-BI(Transient)	5842 $\pm$ 82	59445 $\pm$ 1099
NPC1L1 (Transient)	4092 $\pm$ 377	47026 $\pm$ 2658
SR-BI/NPC1L1 (trans)	3833 $\pm$ 158	52132 $\pm$ 3071
	Cholesteryl Ester, $\pm$ sem	
	dpm	dpm/mg protein
	4 Hour Pulse	
CHO Control (mock)	2132 $\pm$ 40	20497 $\pm$ 640
SR-BI(Transient)	5918 $\pm$ 237	51812 $\pm$ 1417
NPC1L1 (Transient)	1944 $\pm$ 93	19788 $\pm$ 642
SR-BI/NPC1L1 (trans)	4747 $\pm$ 39	58603 $\pm$ 1156
	Free Cholesterol, $\pm$ sem	
	dpm	dpm/mg protein
	4 Hour Pulse	
CHO Control (mock)	45729 $\pm$ 328	439346 $\pm$ 5389
SR-BI(Transient)	50820 $\pm$ 2369	444551 $\pm$ 9785
NPC1L1 (Transient)	39913 $\pm$ 1211	406615 $\pm$ 6820
SR-BI/NPC1L1 (trans)	37269 $\pm$ 1225	459509 $\pm$ 6195

**Example 21: Expression of rat, mouse and human NPC1L1.**

- 5 In this example, *NPC1L1* was introduced into cells and expressed. Species specific NPC1L1 expression constructs were cloned into the plasmid pCDNA3 using clone specific PCR primers to generate the ORF flanked by appropriate restriction sites compatible with the polylinker of the vector. For all three species of NPC1L1, small intestine total tissue RNA was used as a template for
- 10 reverse transcriptase-polymerase chain reaction (RT-PCR) using oligo dT as the

template primer. The rat *NPC1L1* was cloned as an EcoRI fragment, human *NPC1L1* was cloned as a XbaI/NotI fragment and mouse *NPC1L1* was cloned as an EcoRI fragment. Forward and reverse strand sequencing of each clone was performed to confirm sequence integrity. Standard transient transfection procedures were used with CHO cells. In a 6-well plate CHO cells were plated 1 day before transfection at a plating density of  $2 \times 10^5$  cells/well. The following day, cells were incubated with 2  $\mu$ g plasmid DNA and 6  $\mu$ L Lipofectamine for 5 hours followed a fresh media change. Forty-eight hours later, cells were analyzed for NPC1L1 expression using anti-NPC1L1 antisera by either FACS or western blot. To establish stable long term cell lines expressing NPC1L1, transfected CHO cells were selected in the presence of geneticin (G418, 0.8 mg/ml) as recommended by the manufacturer (Life Technologies). Following one month of selection in culture, the cell population was stained with anti-NPC1L1 antisera and sorted by FACS. Individual positive staining cells were cloned after isolation by limiting dilution and then maintained in selective media containing geneticin (0.5 mg/ml).

Other cell types less susceptible to transfection procedures have been generated using adenoviral vector systems. This system used to express NPC1L1 is derived from Ad 5, a type C adenovirus. This recombinant replication-defective adenoviral vector is made defective through modifications of the E1, E2 and E4 regions. The vector also has additional modifications to the E3 region generally affecting the E3b region genes RIDa and RIDb. NPC1L1 expression was driven using the CMV promoter as an expression cassette substituted in the E3 region of the adenovirus. Rat and mouse NPC1L1 were amplified using clone specific primers flanked by restriction sites compatible with the adenovirus vector. Adenovirus infective particles were produced from 293-D22 cells in titers of  $5 \times 10^{10}$  P/mL. Viral lysates were used to infect cells resistant to standard transfection methodologies. In Caco2 cells, which are highly resistant to heterologous protein expression, adenovirus mediated expression of NPC1L1 has been shown by western blot analysis to persist at least 21 days post-infection.

#### Example 22: NPC1L1 Knock-Out Transgenic Mouse.

*NPC1L1* knockout mice were constructed via targeted mutagenesis. This methodology utilized a targeting construct designed to delete a specific region of the mouse *NPC1L1* gene. During the targeting process the *E. coli lacZ* reporter gene

was inserted under the control of the endogenous *NPC1L1* promoter. The region in *NPC1L1* (SEQ ID NO: 45) being deleted is from nucleotide 790 to nucleotide 998. The targeting vector contains the LacZ-Neo cassette flanked by 1.9 kb 5' arm ending with nucleotide 789 and a 3.2 kb 3' arm starting with nucleotide 999. Genomic DNA from the recombinant embryonic stem cell line was assayed for homologous recombination using PCR. Amplified DNA fragments were visualized by agarose gel electrophoresis. The test PCRs employed a gene specific primer, which lies outside of and adjacent to the targeting vector arm, paired with one of three primers specific to the LacZ-Neo cassette sequence. For 5' PCR reconfirmation, the *NPC1L1* specific oligonucleotide ATGTTAGGTGAGTCTGAACCTACCC (SEQ ID NO: 46) and for 3' PCR reconfirmation the *NPC1L1* specific oligonucleotide GGATTGCATTTCTTCAAGAAAGCC (SEQ ID NO: 47) were used. Genotyping of the F2 mice was performed by multiplex PCR using the *NPC1L1* specific forward primer TATGGCTCTGCCCTCTGCAATGCTC (SEQ ID NO: 48) the LacZ-Neo cassette specific forward primer TCAGCAGCCTCTGTTCCACATACACTTC (SEQ ID NO: 49) in combination with the *NPC1L1* gene specific reverse primer GTTCCACAGGGTCTGTGGTGAGTTC (SEQ ID NO: 50) allowed for determination of both the targeted and endogenous alleles. Analysis of the PCR products by agarose gel electrophoresis distinguished the wild-type, heterozygote and homozygote null mouse from each other.

**Example 23: Acute Cholesterol Absorption in *NPC1L1*-Deficient Mice.**

To determine whether *NPC1L1* plays a role in cholesterol absorption, *NPC1L1* deficient mice were studied.

Mice deficient in *NPC1L1* (-/-) were generated by breeding heterozygote mice (+/) to obtain wild-type (+/+) and *NPC1L1* deficient mice (-/-). Non-fasted mice (6.5-9 weeks old, mixed 129 and C57BL/6 background) were weighed and grouped (n=2 -/- and n=4 +/+). All animals were gavaged (Feeding needles, 24G x 1 inch, Popper and Sons, NY) with 0.1 ml corn oil (Sigma; St. Louis, MO) containing 1  $\mu$ Ci  $^{14}$ C-cholesterol (New England Nuclear, [ $^{4-14}$ C] Cholesterol, NEC-018) and 0.1mg carrier cholesterol mass (Sigma; St. Louis, MO). Two hours later, blood was collected by heart puncture. The liver was removed, weighed, and three samples were placed into 20 ml counting vials. Tissues were digested in 1 ml of 1N NaOH at 60°C overnight. The tissue digests were acidified by addition of 250  $\mu$ l



of 4N HCl prior to liquid scintillation counting (LSC). Plasma was isolated by centrifugation at 10,000 rpm for 5 minutes in a microfuge and duplicate 100 $\mu$ l aliquots of plasma were taken for LSC.

Cholesterol absorption, evaluated by this acute technique and expressed as the total amount of radioactive cholesterol in the plasma and liver, demonstrated that the wild type mice (+/+) absorbed an average of 11,773 dpm and *NPC1L1* deficient mice absorbed 992 dpm of the <sup>14</sup>C-cholesterol. These results indicate that the *NPC1L1* deficient mice have a 92% reduction in cholesterol absorption. These data confirm the role of NPC1L1 in intestinal cholesterol absorption. Inhibition of NPC1L1-mediated cholesterol absorption, in a subject, by administering NPC1L1 antagonists, such as ezetimibe, to the subject, are a useful way to reduce serum cholesterol levels and the occurrence of atherosclerosis in the subject.

**Example 24: Cholesterol Absorption in NPC1L1 (NPC3) Knockout Mice (Fecal Ratio Method: Cholesterol/Sitostanol).**

In this example, cholesterol absorption and the activity of ezetimibe was determined in the *NPC1L1* knockout mice (-/-), heterozygous mice (+/-), and age matched wild-type mice (+/+).

Cholesterol absorption in the mice was determined by the dual fecal isotope ratio method as described by Altmann *et al.* (Biochim. Biophys. Acta. 1580(1): 77-93 (2002)). Mice (n= 4-6/group) were fed a standard rodent chow diet and in some groups treated daily with a maximally effective dose of ezetimibe (10 mg/kg). Mice were gavaged with <sup>14</sup>C-cholesterol (1 $\mu$ Ci, 0.1mg unlabeled cholesterol) and <sup>3</sup>H-sitostanol (2 $\mu$ Ci) in 0.1ml corn oil. Feces were collected for 2 days and fecal <sup>14</sup>C-cholesterol and <sup>3</sup>H-sitostanol levels were determined by combustion in a Packard Oxidizer. The fraction of cholesterol absorbed, as evaluated by the fecal dual isotope technique, was similar in wild type (+/+) and heterozygous mice (+/-) fed a chow diet (heterozygous mice absorbed 46  $\pm$  5% and age matched wild type mice absorbed 51  $\pm$  3% of the dose of <sup>14</sup>C-cholesterol). The *NPC1L1* knockout mice (-/-) absorbed 15.6  $\pm$  0.4% of the <sup>14</sup>C-cholesterol, which was similar to the wild type mice treated with a maximally effective dose of ezetimibe (16.1  $\pm$  0.3%), and reduced by 69% compared to wild type mice (p<0.001). In *NPC1L1* knockout treated with ezetimibe at 10 mg/kg/day, cholesterol absorption was similar to that seen in the untreated knockout mice (16.2  $\pm$  0.6% compared to 15.6%  $\pm$  0.4%, respectively). Thus, the majority of

cholesterol absorption is dependent on the presence of NPC1L1 and the residual cholesterol absorption in mice lacking NPC1L1 is insensitive to ezetimibe treatment. These results indicate that NPC1L1 is involved in the small intestinal enterocyte uptake and absorption of cholesterol and is in the ezetimibe sensitive pathway.

5 **Example 25: Mouse Screening Assay (Acute Cholesterol Absorption).**

The following screening assay is used to identify the presence of an NPC1L1 antagonist in a sample.

Mice deficient in *NPC1L1* (-/-) are generated by breeding heterozygote mice (+/) to obtain wild-type (+/+) and *NPC1L1* deficient mice (-/-).

10 In a first set of experiments, non-fasted mice (6.5-9 weeks old, mixed 129 and C57BL/6 background) are weighed and grouped (n=1 to 4 -/- and n=1 to 4 +/+). All animals are gavaged (Feeding needles, 24G x 1 inch, Popper and Sons, NY) with 0.1 ml corn oil (Sigma; St. Louis, MO) containing 1 $\mu$ Ci <sup>14</sup>C-cholesterol (New England Nuclear, [<sup>4-14</sup>C] Cholesterol, NEC-018) and 0.1mg carrier cholesterol mass  
15 (Sigma; St. Louis, MO).

In another set of experiments, 1 to 4 wild-type *NPC1L1* mice (+/+) are treated identically to the mice in the first set of experiments, above, except that the mice are additionally fed a sample to be tested for the presence of an NPC1L1 antagonist.

20 Two hours later, blood is collected from each mouse by heart puncture. The liver is removed, weighed, and three samples are placed into 20 ml counting vials. Tissues are digested in 1 ml of 1N NaOH at 60°C overnight. The tissue digests are acidified by addition of 250 $\mu$ l of 4N HCl prior to liquid scintillation counting (LSC). Plasma is isolated by centrifugation at 10,000 rpm for 5 minutes in a  
25 microfuge and duplicate 100 $\mu$ l aliquots of plasma are taken for LSC.

Cholesterol absorption, evaluated by this acute technique is expressed as the total amount of radioactive cholesterol in the plasma and liver. The sample tested is determined to contain an NPC1L1 antagonist when the level of cholesterol absorption (as measured by the above described methods) in the wild-type *NPC1L1*  
30 mouse (+/+) which was fed the sample and in the *NPC1L1* deficient mouse (-/-) are less than the amount of cholesterol absorption in the wild-type *NPC1L1* mouse (+/+) which was not fed the sample.

**Example 26: Mouse Screening Assay  
(Fecal Ratio Method: Cholesterol/Sitostanol).**

The following screening assay is used to identify the presence of an NPC1L1 antagonist in a sample.

5                   Cholesterol absorption in the mice is determined by the dual fecal isotope ratio method as described by Altmann *et al.* (Biochim. Biophys. Acta. 1580(1): 77-93 (2002)).

                  Three groups of mice (n=1-6/group) are assembled. Two separate groups comprise wild-type *NPC1L1* mice (+/+) and one group comprises *NPC1L1*  
10   deficient mice (-/-).

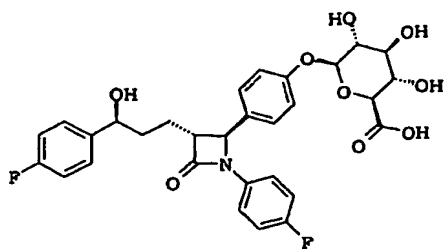
                  Each group is fed a standard rodent chow diet and in some groups treated daily. Mice are gavaged with <sup>14</sup>C-cholesterol (1μCi, 0.1mg unlabeled cholesterol) and <sup>3</sup>H-sitostanol (2μCi) in 0.1ml corn oil. One group of mice, which  
15   comprise wild-type NPC1L1 mice (+/+) are further fed a sample to be tested for the presence of an NPC1L1 antagonist. Feces are collected for 2 days and fecal <sup>14</sup>C-cholesterol and <sup>3</sup>H-sitostanol levels are determined by combustion in a Packard Oxidizer.

                  The sample tested is determined to contain an NPC1L1 antagonist when the level of cholesterol and/or sitostanol absorption (as measured by the above  
20   described methods) in the wild-type *NPC1L1* mouse (+/+) which was fed the sample and in the *NPC1L1* deficient mouse (-/-) are less than the amount of cholesterol and/or sitostanol absorption in the wild-type *NPC1L1* mouse (+/+) which was not fed the sample.

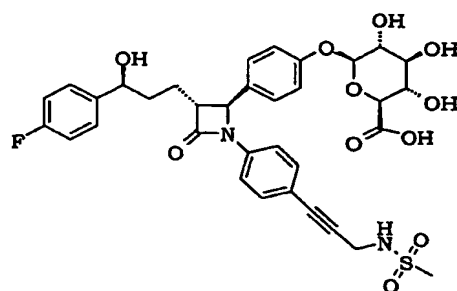
**Example 27: Binding Analysis Using Brush Border Membrane Vesicles**

25                   The following screening assay may be used to identify the presence of an NPC1L1 ligand in a sample.

**Materials.** The following two compounds were synthesized for the binding assay described herein, <sup>3</sup>H-ezetimibe glucuronide **1** (34.5 Ci/mmol) and its  
30   <sup>35</sup>S-propargyl-sulfonamide derivative **2** (800-1100 Ci/mmol).



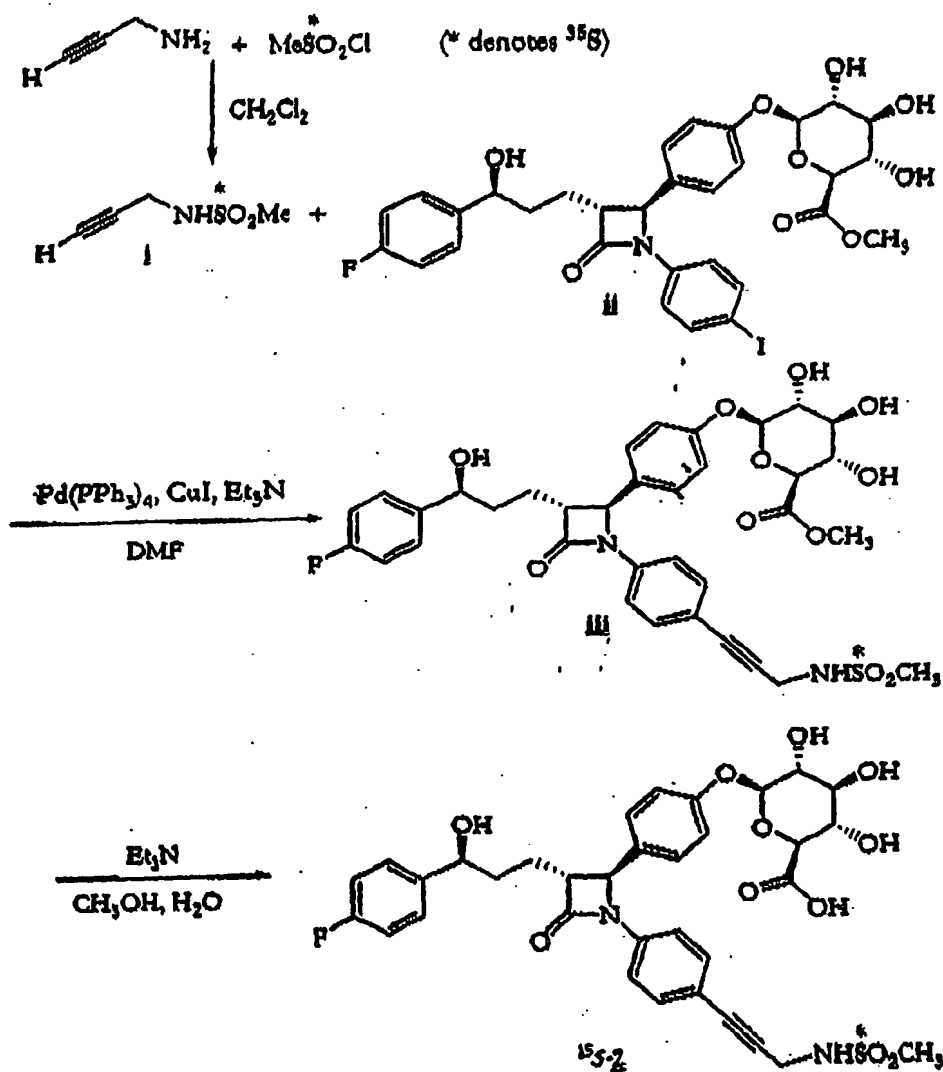
Ezetimibe-glucuronide  
**1**



S-propargyl-sulfonamide  
ezetimibe-glucuronide  
**2**

#### Synthesis of ezetimibe glucuronide and S-propargyl-sulfonamide

- ezetimibe-glucuronide.** Ezetimibe glucuronide (compound **1**) (also referred to as
- 5 EZE-glucuronide) can be made according to the procedures in U.S. Patent No. 5,756,470. The general scheme below illustrates a method for the synthesis of compound **2** and radiolabeled  $^{35}\text{S}$ -**2**.



Preparation of compound  $^{35}\text{S}$ -2 (Compound 2 with radiolabelled  $^{35}\text{S}$ )

Step A: Preparation of [ $^{35}\text{S}$ ]N-prop-2-yn-1-ylmethanesulfonamide (i).

The appropriate volume of [ $^{35}\text{S}$ ]methane sulfonyl chloride (see Dean, D.C.; et al., *J. Med. Chem.* 1996, 39, 1767) totaling 3.5 mCi was removed from a stock solution in methylene chloride and placed in a 5mL conical flask. It was then distilled at atmospheric pressure until the volume was approximately 50  $\mu\text{L}$ . To this solution was immediately added 50  $\mu\text{L}$  of propargylamine. After 15 min, the reaction mixture was diluted with 10 mL of ethyl acetate, washed with saturated sodium bicarbonate solution (3 x 2 mL), and dried over sodium sulfate. After filtration the resulting solution had a count of 3.3 mCi and a radiochemical purity of 99.9 % by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 5 % acetonitrile:H<sub>2</sub>O (0.1 % TFA) to 100 % acetonitrile, 15 min linear gradient, 1 mL/min,  $t_R$  = 4.4 min).

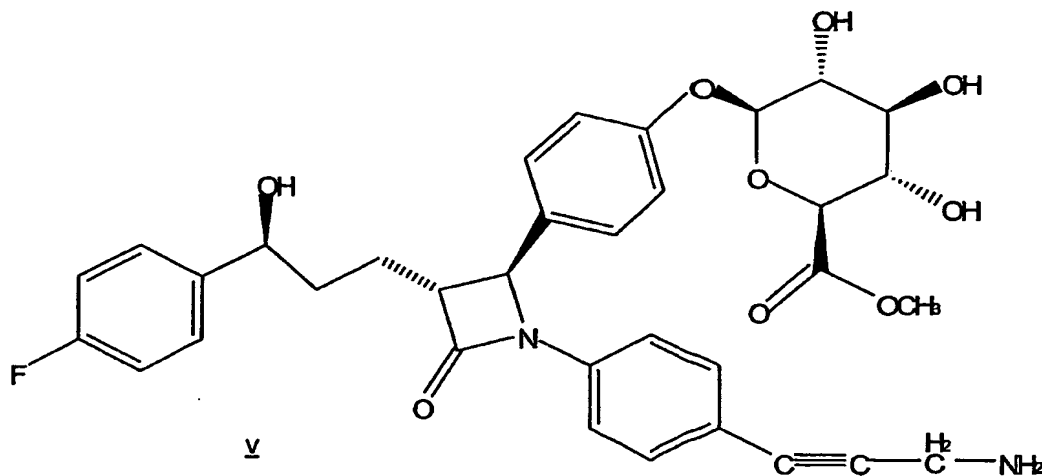
Step B: Preparation of [ $^{35}\text{S}$ ] 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl methyl  $\beta$ -D-glucopyranosiduronate ([ $^{35}\text{S}$ ]) (iii). Dissolved 3.0 mCi of [ $^{35}\text{S}$ ]N-prop-2-yn-1-ylmethanesulfonamide, 1 mg of compound ii (prepared according to Burnett, D.S. et al., *Bioorg. Med. Chem. Lett.* (2002), vol. 12, p. 311), and 1  $\mu\text{L}$  of triethylamine in 100  $\mu\text{L}$  of dimethylformamide inside a plastic microcentrifuge tube. To this solution was added 10  $\mu\text{L}$  of a stock solution containing 8.1 mg of tetrakis(triphenylphosphine)palladium(0) and 1.4mg of copper iodide in 1 mL of dimethylformamide. Stirred at room temperature for sixty hours at which time HPLC indicated 55% conversion. This reaction mixture, which had a radiochemical purity of 44.4% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 5 % acetonitrile:H<sub>2</sub>O (0.1 % TFA) to 100 % acetonitrile, 15 min linear gradient, 1 mL/min,  $t_R$  = 9.3 min) was taken on directly to the next step.

Step C: Preparation of [ $^{35}\text{S}$ ] 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl  $\beta$ -D-glucopyranosiduronic acid  $^{35}\text{S}$ -2. The crude reaction mixture containing compound iii was treated with 25  $\mu\text{L}$  of methanol, 90  $\mu\text{L}$  of water, and 30  $\mu\text{L}$  of triethylamine and stirred at room temperature for one hour at which time it was concentrated to near dryness under a slow stream of nitrogen. The residue was dissolved in 1:1 acetonitrile:H<sub>2</sub>O and subjected to semi-preparative chromatography

- (Zorbax XDB C8 250 x 9.4 mm column, 70:30 acetonitrile:H<sub>2</sub>O (0.1 % TFA) 4 mL/min, 1 x 0.2 mL injections). 540  $\mu$ Ci of product was obtained which had a radiochemical purity of 99.9% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 70:30 acetonitrile:H<sub>2</sub>O (0.1 % TFA), 1 mL/min,  $t_R$  = 10.4 min) and coeluted with an authentic sample of compound 2. LC/MS  $m/z$  = 508 (product – glucuronide – H<sub>2</sub>O), SA = 769 Ci/mmol.

#### Alternate Preparation of <sup>35</sup>S-2.

- Step A: Preparation of iii. The appropriate volume of [<sup>35</sup>S]methane sulfonyl chloride (see Dean, D.C.; et al., *J. Med. Chem.* 1996, 39, 1767) totaling 1.3 mCi was removed from a stock solution in methylene chloride and placed in a 5mL conical flask. It was then distilled at atmospheric pressure until the volume was approximately 50  $\mu$ L. To this solution was immediately added a solution of 1 mg of y in 5  $\mu$ L of pyridine (freshly distilled over calcium hydride).



15

- The solution was stirred at room temperature for five minutes at which time it was concentrated to near dryness under a slow stream of nitrogen. This reaction mixture, which had a radiochemical purity of 80.1% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 5% acetonitrile:H<sub>2</sub>O (0.1 % TFA) to 100 % acetonitrile, 15 min linear gradient, 1 mL/min,  $t_R$  = 9.3 min) was taken on directly to the next step.

Step B: Preparation of <sup>35</sup>S-2. The crude reaction mixture containing iii was treated with 25  $\mu$ L of methanol, 90  $\mu$ L of water, and 30  $\mu$ L of triethylamine and

stirred at room temperature for one hour at which time it was concentrated to near dryness under a slow stream of nitrogen. The residue was dissolved in 1:1 acetonitrile:H<sub>2</sub>O and subjected to semi-preparative chromatography (Zorbax XDB C8 250 x 9.4 mm column, 70:30 acetonitrile:H<sub>2</sub>O (0.1 % TFA) 4 mL/min, 1 x 0.2 mL injections). 350 µCi of product was obtained which had a radiochemical purity of 98.4 % by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 70:30 acetonitrile:H<sub>2</sub>O (0.1 % TFA), 1 mL/min,  $t_R$  = 10.4 min) and coeluted with an authentic sample of 2. LC/MS  $m/z$  = 508 (product – glucuronide – H<sub>2</sub>O), SA = 911 Ci/mmol.

Following the same general procedure for synthesis of <sup>35</sup>S-2, except omitting the radiolabel ling, compounds 2 and iv can be prepared.

#### Preparation of brush border membrane vesicles (BBMV).

Membranes were prepared from Rhesus macaque (*Macaca mulatta*), rat (male Sprague-Dawley), and mouse (male C57BL/6J) intestines, using Mg<sup>++</sup> precipitation method described in the following references and with modifications described below (Hauser, H., Howell, K., Dawson, R.M.C., Bowyer, D. E. *Biochim. Biophys. Acta* 602, 567-577 (1980); Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal, K., Muller, G., Tripier, D., Wess, G. *J. Biol. Chem.* 268, 18035-18046 (1993); Rigtrup, K.M., Ong, D.E. *Biochemistry* 31, 2920-2926 (1992)).

The intestines from freshly sacrificed animals were cut into segments, perfused with ice-cold saline buffer (Buffer A: 26 mM NaHCO<sub>3</sub>, 0.96 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM HEPES, 5.5 mM glucose, 117 mM NaCl, 5.4 mM KCl, pH = 7.4), placed on cold glass plates, opened longitudinally, and the mucosa scraped with glass microscope slips. This mucosa could be used fresh or frozen with identical results. To prepare the membranes, the mucosal scrapings were resuspended in 20 volumes of cold buffer consisting of 300 mM D-mannitol, 5 mM EGTA, 12 mM Tris, pH 7.4 with HCl, and containing 0.1 mM PMSF and a 1% dilution of a protease inhibitor cocktail (set 1, Calbiochem). They were homogenized using a Polytron at medium speed on ice until inspection with a microscope indicated complete cell lysis. Then, solid MgCl<sub>2</sub> was added slowly with stirring to a final concentration of 10 mM, and the solution was kept stirring on ice for 15 min. Cellular debris was removed by centrifugation for 15 min at 3,000g, and the membranes were recovered by centrifugation for 60 min at 48,000g. The membranes were further rinsed by re-suspension in a buffer containing 50 mM D-mannitol, 5 mM EGTA, and 2 mM Tris



at pH 7.40, and centrifugation for 60 min at 48,000g. The final pellet was resuspended in 120 mM NaCl and 20 mM Tris at pH 7.40 to a concentration of ~10-20 mg protein/ml, aliquoted, frozen in liquid nitrogen, and stored at -80C. The activity was stable indefinitely and could be freeze-thawed with minimal loss of activity.

Membrane protein was measured by the Bradford assay (Bradford, M.M. Anal. Biochem. 72, 248-254 (1976)) using bovine serum albumin as standard. The enrichment in brush border membranes was assessed using gamma-glutamyltransferase as a marker enzyme (Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal, K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. 268, 18035-18046 (1993)), which indicated a 6-fold enrichment over the initial homogenate.

**Binding assay.** Assays were conducted in 12 x 75 mm glass test tubes and total volume 20-100 ul. In general, frozen membranes were diluted in buffer A or buffer A containing 0.03% taurocholate and 0.05% digitonin to a final concentration of 0.02 to 5 mg/ml. Radiolabeled ligands were typically 25-50 nM for <sup>3</sup>H-ezetimibe (EZE)glucuronide 1, and 3-5 nM for its <sup>35</sup>S analog 2, in the assay, and they were delivered as DMSO or CH<sub>3</sub>CN solutions. Competing ligands were likewise added as DMSO solutions to give a total 2-10 % organic solvent content. Nonspecific binding was defined by competition with 100 uM ezetimibe glucuronide. At least 2 components of buffer A, the bicarbonate and phosphate salts, were later found to be inconsequential and were routinely omitted. To ensure equilibrium was established, the reactions with compound 1 were incubated at least 3 hours for rhesus membranes and at least one hour for rat membranes at room temperature, and with compound 2 as long as 2 hours at 37°C with rhesus and rat brush membranes. Additionally, reactions with compound 2 were incubated as long as 2 hours at 37°C with membranes from HEK-293 cells expressing mouse, rat or human NPC1L1.

Bound ligand was quantified by single-tube vacuum filtration using GF/C glass fiber filters. Glass fiber filters (GF/C) were obtained from Whatman. The filters were pretreated by soaking with 0.5% polyethylenimine to reduce nonspecific binding. Filtration was accomplished by adding 2.5 ml of ice cold buffer (120 mM NaCl, 0.1% sodium cholate, and 20 mM MES at pH 6.70) to the assay tube, pouring the mixture through the filter, and then rinsing the tube and filter twice more with

another 2 x 2.5 ml buffer. The filters were counted in 7 ml vials using Packard DM liquid scintillation fluid. Where triplicate assays were performed, the standard error was typically <4%. As an example, a 100µl assay of rat brush border membranes at 2 mg/ml in the presence of 400,000 dpm (50 nM) <sup>3</sup>H-ezetimibe glucuronide gave  
5 15,000 dpm specific and 3,000 dpm nonspecific binding. The filters contributed most of the nonspecific binding (2,000 dpm).

Alternatively, vacuum filtration of compound 2 on a Millipore 96-well plate (Whatman GF/C) can also be used to achieve adequate precision.

**Data analysis.** Data from saturation experiments were subjected to a  
10 Scatchard analysis, and linear regression was performed to yield the equilibrium dissociation constant ( $K_d$ ) and maximum receptor concentration ( $B_{max}$ ). Correlation coefficients for these determinations were typically greater than 0.96. Data from competition experiments were analyzed and  $IC_{50}$  values determined from Hill plots of the binding data. The kinetic data for ligand association and dissociation were  
15 subjected to the analysis of Weiland and Molinoff (Weiland, G., Molinoff, J.B. Life Sci. 29, 313-330 (1981)). The dissociation rate constant for ( $k_{-1}$ ) was determined directly for a first order plot of ligand dissociation versus time. The rate of ligand association ( $k_1$ ) was determined from the equation  $k_1 = k_{obs}([LR_e]/([L] [LR]_{max}))$  where  $[L]$  is the concentration of ligand,  $[LR_e]$  is the concentration of the complex at  
20 equilibrium,  $[LR]_{max}$  is the maximum number of receptors present, and  $k_{obs}$  is the slope of the pseudo-first order plot  $L_n ([LE]_0/([LR]_e - [LR]_t))$  versus time.

**Binding analysis.** Ezetimibe is rapidly converted to its glucuronide *in vivo*, and this metabolite is thought to be largely if not exclusively responsible for inhibition of cholesterol uptake. Accordingly, both <sup>3</sup>H-ezetimibe and its  
25 corresponding glucuronide derivative (1) were prepared and tested for binding to intestinal brush border membrane preparations, using a single-tube vacuum filtration technique. As a result of the hydrophobic nature of <sup>3</sup>H-ezetimibe, high nonspecific binding was observed, precluding its use as a radioligand for the binding assay. However, due to the improved physical properties of the glucuronide derivative (1),  
30 specific binding was observed with this radioligand and it was used to assess binding in rhesus, rat, and mouse intestinal brush border membranes.

Rhesus, rat, and mouse intestinal scrapings were homogenized and the brush border membranes isolated. Specific binding was observed exclusively in the

membrane fraction. Plots of total, nonspecific, and specific binding to rhesus (Figure 1) and rat (Figure 2) brush border membranes. Aliquots of rhesus BBMV (83  $\mu\text{g}/\text{assay}$ ) or rat BBMV (250  $\mu\text{g}/\text{assay}$ ) were incubated with increasing concentrations of  $^3\text{H}$ -EZE-glucuronide. Total binding and nonspecific binding determined in the presence of 10-100  $\mu\text{M}$  EZE-glucuronide are shown. Specific binding was calculated from the difference between total and nonspecific binding. Data were fit by nonlinear regression as described above, and the linear Scatchard plot is shown. In rhesus membranes, the data correspond to a single binding site with  $K_d = 41 \text{ nM}$  and a concentration of 5.5 pmol/mg membrane protein. The affinity is  $\sim 10$ -fold lower in rat membranes ( $K_d = 540 \text{ nM}$ ).  $^3\text{H}$ -EZE-glucuronide is not the best ligand for a binding assay for the mouse target due to the compounds low affinity in mouse membrane. These potencies correlate roughly with the sensitivity of these species to ezetimibe inhibition of cholesterol uptake.

**Rate constants for binding and dissociation.** Ezetimibe-glucuronide is slow to bind, and forms a relatively long-lived complex with its receptor. Indeed, this was key to detecting the interaction in a traditional filter-binding assay, as ligand/receptor interactions with  $K_d$  values greater than 100 nM often go unrecognized because of the typical fast off-rates of the ligands. Rate constants for association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) of compound **1** were determined for rat and rhesus membranes, and used as an alternative method to calculate the dissociation constant ( $K_d$ ) according to the relationship  $K_d = k_{off}/k_{on}$ . 300  $\mu\text{g}/\text{assay}$  of rat brush border membrane vesicles were incubated with 25 nM  $^3\text{H}$ -EZE-glucuronide at room temperature for up to three hours for the association kinetic studies. 83  $\mu\text{g}/\text{assay}$  of rhesus brush border membrane vesicles were incubated with 25 nM  $^3\text{H}$ -EZE-glucuronide at room temperature for up to five hours for the association kinetic studies. Nonspecific binding measured in the presence of 100  $\mu\text{M}$  EZE-glucuronide was subtracted from the total binding to calculate the specific binding shown in Figures 3A and 4A. For the dissociation kinetic study, rat brush border membrane vesicles were incubated with 25 nM  $^3\text{H}$ -EZE-glucuronide for 2 hours at room temperature and ligand dissociation was initiated by the addition of 100  $\mu\text{M}$  EZE-glucuronide. Rhesus brush border membrane vesicles were incubated with 42 nM  $^3\text{H}$ -EZE-glucuronide for 4 hours at room temperature and ligand dissociation was initiated by the addition of 100  $\mu\text{M}$  EZE-glucuronide. For both rat and rhesus

dissociation studies, samples were collected at various times and radiolabel was detected. Dissociation curves are shown in Figures 3B (rat) and 4B (rhesus).

For rat membranes, the rate constant for association is  $k_{on} = 5,540 \text{ M}^{-1} \text{ s}^{-1}$  (compared to  $10^8$  to  $10^9 \text{ M}^{-1} \text{ s}^{-1}$  for diffusion controlled encounter), and the rate constant for dissociation is  $k_{off} = 2.4 \times 10^{-3} \text{ s}^{-1}$ , corresponding to a half-life of 4.7 min. The data are shown in Figure 3, where the solid lines are theoretical for these rate constants. The  $K_d$  value predicted from these rate constants ( $K_d = k_{off}/k_{on} = 440 \text{ nM}$ ) agrees well with that measured at equilibrium ( $K_d = 540 \text{ nM}$ ).

For rhesus membranes, where  $^3\text{H}$ -ezetimibe glucuronide is at least 10-fold more potent (as described above), the association rate remains the same but the half-life for dissociation of the complex increases to ~90 min. These data are shown in Figure 4, where the theoretical lines correspond to  $k_{on} = 3,900 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_{off} = 1.23 \times 10^{-4} \text{ s}^{-1}$ , and predict  $K_d = 32 \text{ nM}$  compared to that measured at equilibrium ( $K_d = 41 \text{ nM}$ ).

#### Example 28: Binding Analysis of a Potent NPC1L1 ligand

A  $^{35}\text{S}$ -labeled propargyl-sulfonamide analogue of ezetimibe glucuronide ( $^{35}\text{S}$ -2) was identified as a potential NPC1L1 antagonist. Compound 2 was prepared as described in Example 27 and found to have markedly improved affinity for some species of brush border membranes vesicles. For rhesus brush border membranes vesicles, 56  $\mu\text{g}$  protein/assay were incubated with 25 nM  $^3\text{H}$ -EZE-glucuronide in the presence of increasing concentrations of EZE-glucuronide and 2. For rat brush border membranes vesicles, 150  $\mu\text{g}$  protein/assay were incubated with 50 nM  $^3\text{H}$ -EZE-glucuronide in the presence of increasing concentrations of EZE-glucuronide and 2. For mouse brush border membranes vesicles, 20  $\mu\text{g}$  protein/assay were incubated with 3 nM  $^{35}\text{S}$ -2 in the presence of increasing concentrations of EZE-glucuronide and 2.

2 is more potent against enterocyte brush border membrane preparations from rats (35-fold), but is equipotent with ezetimibe glucuronide for rhesus membrane preparations (Figure 5, Table 8). It also has enhanced affinity for mouse membranes (Figure 6, Table 8).

**Table 8. Summary of inhibition constants ( $K_i$ ) for binding of ezetimibe glucuronide 1 and its propargyl-sulfonamide derivative 2 to rhesus, rat, and mouse intestinal brush border membranes.**

Compound	Rhesus	Rat	Mouse
<u>1</u>	39	530	2,300
<u>2</u>	38	15	144

5  $K_i$  values are nM.

**Example 29: Distribution of  $^3\text{H}$ -ezetimibe glucuronide (1) binding to intestinal tissues.**

Previous studies have established that cholesterol absorption occurs primarily in the jejunum, and is substantially lower in the ileum and duodenum. To  
 10 determine if the binding activity is similarly distributed, the binding assay using  $^3\text{H}$ -ezetimibe glucuronide ( $^3\text{H}$ -1) as a radioligand was used to determine the distribution of binding sites in sections from rhesus and rat intestines.

For the rhesus studies, 10 cm corresponding to the ileum of a rhesus small intestine was separated and the remaining intestine was divided into three  
 15 segments, (proximal, middle and distal) of equal length (70 cm each). For the rat studies, 10 cm corresponding to the ileum of a rhesus small intestine was separated and the remaining intestine was divided into three segments, (proximal, middle and distal) of equal length (36 cm each). Brush border membrane vesicles were prepared as described in Example 27. Aliquots of vesicles (100-200 $\mu\text{g}$ ) protein/assay were  
 20 incubated with 50nM  $^3\text{H}$ -EZE-glucuronide in the absence and presence of 100 $\mu\text{M}$  EZE-glucuronide.

As shown in Figure 7, specific binding for  $^3\text{H}$ -ezetimibe glucuronide peaks in the jejunum in both species, consistent with the previously observed pattern of cholesterol absorption.

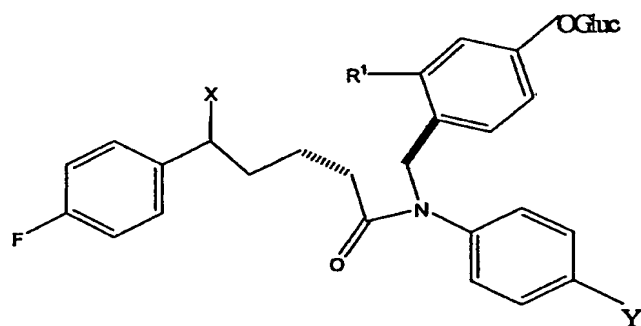
25 **Example 30: Correlation of *in vitro* and *in vivo* binding activity of NPC1L1**

To determine if *in vitro* binding activity is predictive of *in vivo* efficacy, the enantiomer of ezetimibe glucuronide and several close structural analogues of ezetimibe glucuronide that were tested in the rat membrane binding assay were tested in an acute rat cholesterol absorption study as described in  
 30 Examples 23-26. The selected analogs had a range of *in vitro* potencies, and were

anticipated to have similar physical properties to ezetimibe glucuronide (Tables 9 and 12). The enantiomer, which has a  $K_d > 100,000$  nM for the rat target, was inactive in the *in vivo* assay. For the other analogs, the same rank order of potency is observed in the *in vitro* and *in vivo* assays, further evidence that the observed binding is due to the target of ezetimibe.

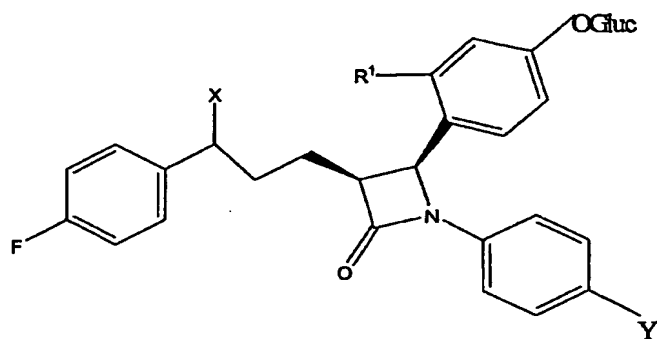
**Table 9.  $IC_{50}$  values of EZE-gluc and analogs to inhibit binding of 3H-EZE-gluc to rat brush border membrane vesicles.**

Compound Name	R <sup>1</sup>	X	Y	RAT $IC_{50}$ (nM)
<u>3</u>	H	H2	F	2,300
EZE-gluc <u>1</u>	H	(S)-OH	F	530
EZE-gluc enantiomer <u>4</u>	H	(R)-OH	F	>100000
<u>5</u>	H	(R)-OH	F	3,900
<u>6</u>	H	=O	F	70,000
<u>7</u>	OH	(S)-OH	F	252



10

compounds 1, 3, 5, 6 and 7.



Backbone structure for compound 4.

**Example 31: Binding affinities of ezetimibe glucuronide and its analogs to recombinant NPC1L1**

NPC1L1 was identified as a candidate target of ezetimibe from a  
5 search of genetic databases for cholesterol binding motifs. Subsequently, *NPC1L1*  
deficient mice were found to have 80% reduction of cholesterol absorption, and did  
not respond to ezetimibe treatment, strongly suggesting that this protein is required  
for ezetimibe efficacy. To determine if NPC1L1 is the direct target of ezetimibe,  
binding affinities were compared for ezetimibe glucuronide and several analogs to  
10 NPC1L1 transfected cells and rat brush border membrane vesicles.

Rat NPC1L1 transfected CHO cells (~500,000 cells/assay) were  
incubated with 5 nM <sup>35</sup>S-2 (~1 million dpm/assay) for 2 hours at 37 °C in the absence  
or presence of increasing concentrations of EZE-glucuronide (compound 1),  
compounds 2, 3, 5, 6, or 8. Compound 8 is an analog of compound 2 wherein the  
15 hydroxyl group in the 3-hydroxypropyl moiety of 2 is replaced with an oxo group.

Human NPC1L1 transfected CHO cells (~600,000 cells/assay) were  
incubated with 5 nM <sup>35</sup>S-2 (~1 million dpm/assay) in buffer A for 2 hours at 37 °C in  
the absence or presence of increasing concentrations of EZE-glucuronide (compound  
1), compounds 2, 3, 5, 6, or 8.

20 As shown in Figures 9 and 12, and Table 10, the affinities for the  
recombinant and native proteins are virtually identical, providing compelling evidence  
that NPC1L1 is the direct target of ezetimibe in mammalian tissues, and that other  
proteins are not required for binding.

Affinities of ezetimibe glucuronide and analogues thereof were also  
25 determined for human recombinant NPC1L1. The results, shown in Figure 9, indicate  
that ezetimibe glucuronide (1) has an affinity for the human protein of 907 nM. The  
propargyl-sulfonamide analogue (2) is approximately 50-fold more potent, with a  
K<sub>d</sub> = 21 nM, suggesting that this compound has the potential for enhanced potency of  
cholesterol absorption inhibition in man.

**Table 10: Comparison of inhibition constants (K<sub>i</sub>) for binding to native rat intestinal brush border membranes and membranes from rat NPC1L1 transfected cells.**

Analog	Recombinant rat NPC1L1 K <sub>i</sub> , nM	Rat BBMV K <sub>i</sub> , nM
EZE-glucuronide <u>1</u>	790	600
<u>2</u>	12	15
<u>3</u>	2400	2300
<u>6</u>	84500	70000
<u>5</u>	5800	3900
<u>8</u>	556	818

**5 Example 32: Binding of <sup>35</sup>S-2 to membranes from wild type and NPC1L1 deficient mice.**

Final confirmation that NPC1L1 is the target of ezetimibe was provided by binding studies with <sup>35</sup>S-2 in intestinal brush border membranes from NPC1L1 deficient and control mice.

10 Brush border membranes vesicles were prepared from intestinal tissues of wild type and *NPC1L1* knockout (-/-) mice. 15, 30 and 60μg protein/ assay of brush border membranes vesicles were incubated with 4nM <sup>35</sup>S-2 in buffer A for 3 hours at 37°C in the presence and absence of 100μM EZE-glucuronide.

15 The results, shown in Figure 10, indicate that no detectable binding is observed in membranes from NPC1L1 deficient mice, whereas age matched wild type control membranes have detectable binding. The binding affinity observed in this experiment in control mouse membranes (K<sub>d</sub>=156 nM) was virtually identical to that observed in previous studies (Figure 11).

**20 Example 33: Binding Analysis Using Brush Border Membrane Vesicles from Rat Mouse and Rhesus Monkey**

Binding studies were performed to compare the relative binding affinity of ezetimibe glucuronide to various brush border membrane vesicles.



<sup>3</sup>H-ezetimibe glucuronide **1** was prepared as described in Example 27.

The brush border membranes were prepared as described in Example 27.

Binding Assay. Assays were conducted in 12 x 75 mm glass test tubes and total volume 20-100 µl. In general, frozen membranes were diluted in buffer A or  
5 buffer A containing 0.03% taurocholate and 0.05% digitonin to a final concentration of 0.5 to 5 mg/ml (Buffer A: 26 mM NaHCO<sub>3</sub>, 0.96 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM HEPES, 5.5 mM glucose, 117 mM NaCl, 5.4 mM KCl, pH = 7.4). Final concentrations of [<sup>3</sup>H]ezetimibe glucuronide **1** were typically 25-50 nM, and were delivered as DMSO or CH<sub>3</sub>CN solutions. Competing ligands were likewise added as DMSO solutions to  
10 give a total 1-5 % organic solvent content. Nonspecific binding was defined by competition with 100-500 µM ezetimibe glucuronide. At least three components of buffer A, the bicarbonate and phosphate salts, and glucose, were later found to be inconsequential and were routinely omitted. Reactions were incubated until equilibrium was achieved (one hour for rat or three hours for rhesus membranes).

15 Bound ligand was recovered by single-tube vacuum filtration on Whatman GF/C glass fiber filters. The filters were pretreated by soaking with 0.5% polyethylenimine to reduce nonspecific binding. Filtration was accomplished by adding 2.5 ml of ice cold buffer (120 mM NaCl, 0.1 % sodium cholate, and 20 mM MES at pH 6.7) to the assay tube, pouring the mixture through the filter, and then  
20 rinsing the tube and filter twice more with another 2 x 2.5 ml buffer. The filters were counted in 7 ml vials using Ultima Gold MV liquid scintillation fluid from Packard. Where triplicate assays were performed, the standard error was typically <4%. As an example, a 100µl assay of rat brush border membranes at 2 mg/ml in the presence of 400,000 dpm (50 nM) [<sup>3</sup>H]ezetimibe glucuronide gave 15,000 dpm specific and 3,000  
25 dpm nonspecific binding. The filters contributed most of the nonspecific binding (2,000 dpm).

Data Analysis. After correction for nonspecific binding, saturation-binding data were fit by nonlinear regression (Sigma Plot) to the single-site expression  $[B] = B_{\max} \times [L]/([L] + K_D)$ . Linear Scatchard plots are shown for  
30 illustration. Data on K<sub>i</sub> from competition experiments were analyzed by nonlinear regression to the expression  $[B] = [B_0]/(1 + [I]/K_i^{\text{obs}})$ , and where required were corrected for radioligand competition as  $K_i = K_i^{\text{obs}}/(1 + [L^*]/K_D)$ .

First-order rate constants ( $k_{\text{obs}}$  and  $k_{\text{off}}$ ) were determined by nonlinear regression to the first order rate equation  $A = A_0 e^{-kt}$ . Kinetic data for  $k_{\text{on}}$  were analyzed according to Weiland and Molinoff (32), using the equation  $k_{\text{on}} = k_{\text{obs}} ([\text{LR}]_0 / ([\text{L}] [\text{LR}]_{\text{max}}))$ , where  $[\text{L}]$  is the concentration of ligand,  $[\text{LR}]_0$  is the concentration of the complex at equilibrium,  $[\text{LR}]_{\text{max}}$  is the maximum number of receptors present, and  $k_{\text{obs}}$  is the apparent first-order rate constant.

Binding analysis. Binding studies using the [ $^3\text{H}$ ]ezetimibe glucuronide a traditional rapid-filtration assay on glass fiber filters using enterocyte brush border membrane preparations from rat, mouse and rhesus monkey were performed (Table 11). Table 11 shows the binding affinities of [ $^3\text{H}$ ]ezetimibe glucuronide to the membranes in the absence of detergents. The observed binding was specific, saturable, and consistent with a single molecular site. Scatchard analyses and the specific/nonspecific binding windows for rat and monkey are shown in Figure 12. The binding affinity is relatively weak in rat membranes ( $K_D = 542$  nM) and even weaker in murine membranes ( $K_D = 10,000$  nM). In contrast, binding affinity in rhesus monkey membranes is approximately 10-fold greater ( $K_D = 41$  nM). The number of binding sites varied from 5 – 20 pmol/mg membrane protein depending on species and preparation.

The rates for binding and dissociation of [ $^3\text{H}$ ]ezetimibe glucuronide were determined and found to be slow relative to those typically observed for protein-ligand interactions. For example, the rate constants for association to rat and monkey brush border membranes are  $k_{\text{on}} = 5.54$  and  $3.90 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  (Figure 12). These are 100,000-fold smaller than those typically observed for a diffusion controlled encounter,  $10^8$  to  $10^9 \text{ M}^{-1} \text{ s}^{-1}$ . Similarly, these complexes are unusually long-lived, dissociating with rate constants of  $k_{\text{off}} = 2.4 \times 10^{-3} \text{ s}^{-1}$  and  $1.2 \times 10^{-4} \text{ s}^{-1}$  at 25C, equivalent to half-lives of 4.7 and 96 min for the rat and monkey complexes, respectively. In comparison, half lives are normally <1 sec for dissociation of common diffusion controlled, 100 nanopolar  $K_D$  ligands. These rate constants predict  $K_D$  values ( $K_D = k_{\text{off}}/k_{\text{on}}$ ) of 440 and 32 nM, respectively, which agree well with those measured by equilibrium titration (Figure 12), and by saturation as described earlier. Such slow-forming, long-lived complexes suggest that conformational changes in the protein are rate limiting.

**Table 11: Comparison of ezetimibe binding affinity and cross species efficacy**

Species	K <sub>D</sub> (nM)	ED <sub>50</sub> (mg/Kg)
mouse	12,000	0.5000
rat	542	0.0300
monkey	41	0.0005

Table 11 also shows a correlation between *in vitro* and *in vivo* binding of [<sup>3</sup>H]ezetimibe glucuronide in various enterocyte brush border membrane preparations from rat, mouse and rhesus monkey. The *in vivo* ED<sub>50</sub> values are derived from cholesterol absorption and cholesterol feeding studies. The rank order of ezetimibe potency (ED<sub>50</sub>) *in vivo* as follows: rhesus (0.0005mpk) > rat (0.03mpk) > mouse (0.5mpk) is the same as the order of *in vitro* binding affinity (IC<sub>50</sub>) as follows: rhesus monkey (41nM) < rat (542nM) < mouse (12,000nM).

The binding affinities of **1** to brush border membranes correlate well across species with the sensitivity to ezetimibe inhibition of cholesterol uptake *in vivo* (mouse < rat < monkey) (Clader, J. W. The discovery of ezetimibe; A view from outside the receptor. *J Med. Chem.* 47, 1-9 (2004); Davis, H.R. Jr., Compton, D.S., Hoos, L. & Tetzloff, G. Ezetimibe, a potent cholesterol absorption inhibitor, inhibits the development of atherosclerosis in ApoE knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 21, 2032-2038 (2001); Burnett, D.A. Beta-lactam cholesterol absorption inhibitors. *Curr. Med. Chem.* 11, 1873-1887 (2004), consistent with the hypothesis that the assay is relevant to the target of ezetimibe *in vivo* (Table 11). As evidence that this interaction is very specific, the glucuronide of the enantiomer of ezetimibe was prepared and found to be completely inactive *in vitro* (K<sub>i</sub> > 100 x K<sub>D</sub> for ezetimibe glucuronide in all species), consistent with its lack of activity *in vivo* in a rat acute cholesterol absorption model (see Table 12 in which the enantiomer is analyzed).

**Example 34: NPC1L1 as the target of ezetimibe in NPC1L1-expressing HEK293 cells.**

This example demonstrates that ezetimibe binds specifically to NPC1L1-expressing HEK293 cells.

Transient expression of NPC1L1. Plasmid pCR3.1 expressing rat NPC1L1 (Genbank AY437867) or human NPC1L1 (Genbank AY437865) were

prepared using standard molecular biology protocols. HEK-293 cells (ATCC) were seeded at  $10 \times 10^6$  cells per T-225 flask (Corning) in DMEM containing 10% fetal calf serum, 4.5 g/L D-glucose and L-glutamine, 18 hours prior to transfection. They were transiently transfected with 25 µg of DNA using Fugene transfection reagent (Roche Biochemical) at a ratio of 6:1 Fugene:DNA. Following transfection, the cells were incubated at 37°C and 5% CO<sub>2</sub> for 48 hours, and then harvested using PBS based cell dissociation buffer (Gibco), pelleted at 500 x g, snap frozen on dry ice, and stored at -80°C.

Membrane preparation from HEK-293 cells. Membranes were prepared by resuspending the frozen cell pellets in ten volumes of 20 mM HEPES/Tris buffer at pH 7.40 containing 8% sucrose, and sonicating the suspensions with a probe sonicator on ice until most of the cells were lysed. To isolate the membranes, the sonicates were centrifuged at 1600 x g for 10 min to remove cell debris, and then the supernatants were centrifuged at 125,000 x g for 1 hour to recover the membranes. These membranes were resuspended in 20 mM HEPES/Tris buffer at pH 7.40 containing 160 mM NaCl and 5% glycerol, and stored at 10-20 mg/ml protein at -80°C.

Pursuing the recent evidence indicating that NPC1L1 is an important component of the pathway inhibited by ezetimibe, recombinant rat and human NPC1L1 were expressed in human embryonic kidney (HEK) 293 cells (Figure 13, Panel 1). Cell lysates from HEK-293 cells expressing NPC1L1 (Lanes 1 and 3 of Panel 1 Figure 13) and wild-type cells (Lanes 2 and 4 of Panel 1 Figure 13) were analyzed by gel electrophoresis and Western blot with an anti-NPC1L1 antibody A1801. An excess of NPC1L1-specific peptide was included to assess specificity of the antibody for NPC1L1 (Lane 3 and 4 of Panel 1 Figure 13). Preliminary binding studies using 1 revealed specific binding to membrane preparations from cells expressing NPC1L1, and no specific binding to membranes from mock transfected cells (not shown).

Binding to NPC1L1 expressing cells was also observed with a BODIPY-labeled fluorescent ezetimibe glucuronide analog (SCH354909) (Figure 13, Panel 2A). Panel 2 of Figure 13 shows confocal microscope images of a fluorescent ezetimibe glucuronide analog (SCH354909) bound to the surface of NPC1L1-293 cells (Panel 2A), nonspecific binding of SCH354909 to NPC1L1-293 cells in the

presence of 100  $\mu$ M unlabeled ezetimibe glucuronide (Panel 2B), binding of SCH354909 to wild type HEK 293 cells (Panel 2C), and nonspecific binding of SCH354909 to wild type HEK 293 cells in the presence of 100  $\mu$ M unlabeled ezetimibe glucuronide (Panel 2D). In each case, plated cells were incubated in culture media with 500 nM SCH354909 for 4 hours at 37°C. Cells were subsequently washed with PBS and fluorescence was detected using confocal microscopy.

Binding of SCH354909 was clearly evident at the cell surface membrane of the NPC1L1-expressing cells and was completely abolished in the presence of excess unlabeled ezetimibe glucuronide (Figure 13, Panel 2C). No binding was observed in wild type HEK 293 cells (Figure 13, Panels 2B and 2D). These results demonstrated that ezetimibe glucuronide binds specifically to NPC1L1.

**Example 35: NPC1L1 as the *in vivo* target of ezetimibe.**

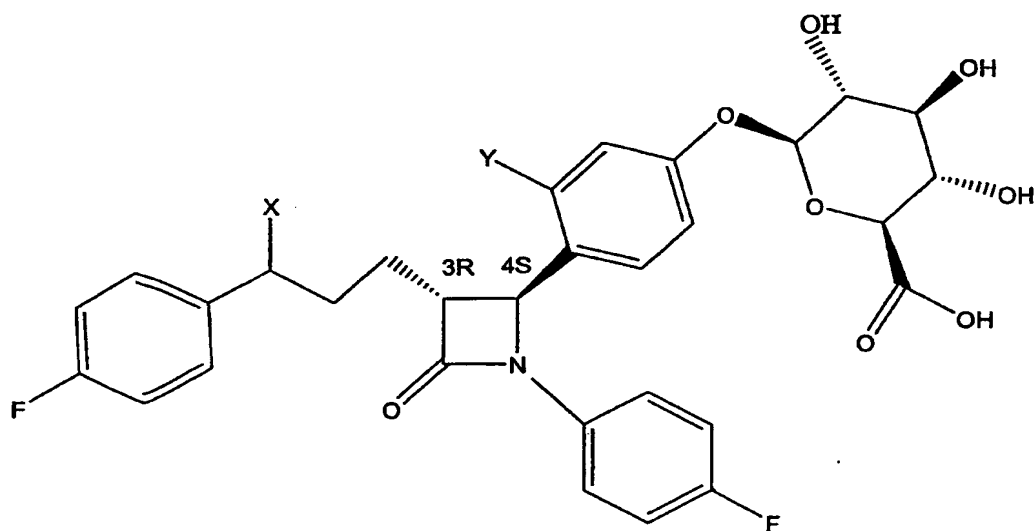
To obtain evidence that NPC1L1 is the direct binding target of ezetimibe *in vivo*, binding affinities of 1 and several key analogs were determined for recombinant rat and human NPC1L1 expressed in HEK-293 cell membranes and compared to those for native rat and rhesus intestinal enterocyte brush border membranes. A series of ezetimibe analogs was selected with subtle structural diversity, but with no binding affinities to native brush border membranes that covered a range of 1000-fold.

Table 12 shows a comparison of binding affinities ( $K_i$  values) for recombinant NPC1L1-293 cell membranes and native brush border membranes. Selected analogs of ezetimibe glucuronide are compared against recombinant rat and human NPC1L1 membranes prepared from transiently transfected HEK-293 cells compared to native rat and rhesus brush border membranes. The binding assays were conducted in a final volume of 20  $\mu$ l in the presence of 0.03% sodium taurocholate and 0.05% digitonin until equilibrium was achieved. 1.25 mg protein/ml and 100 nM 1 were used for native rat, recombinant rat, and recombinant human experiments, and 1.25 mg protein/ml and 20 nM 1 were used for native rhesus monkey experiments. Observed total and nonspecific binding, respectively, in the absence of inhibition were native rat: 7,700 & 1,100, recombinant rat: 33,000 & 1,100, native rhesus monkey: 7,300 & 367, and recombinant human: 19,200 & 1,000 dpm. Analog structures are defined in Table 12. Compound 4 has the stereochemical configuration 3S, 4R, and is the glucuronide of the enantiomer of ezetimibe. These determinations

were conducted in buffer containing 0.03% taurocholate and 0.05% digitonin, levels below the critical micelle concentrations of these detergents. These conditions enhanced apparent binding by as much as 20-fold for the recombinant preparations (principally a  $B_{\max}$  effect), and greatly facilitated a quantitative comparison of  $K_i$  values for 1 and its analogs.

As shown in Table 12, the  $K_i$  values for recombinant rat NPC1L1 and native rat brush border membranes are virtually identical, strongly suggesting that NPC1L1 is the molecular target of ezetimibe *in vivo*. In the case of membranes from cells expressing recombinant human NPC1L1, the binding affinities also parallel those observed in rat membranes, whereas binding affinities for native rhesus brush border membranes are uniformly ~10-fold more potent. This result is consistent with the finding that ezetimibe is an order of magnitude more potent in monkey than in human or rat (Clader, J. W. The discovery of ezetimibe; A view from outside the receptor. *J Med. Chem.* 47, 1-9 (2004); Jeu, L. & Cheng, J.W. Pharmacology and therapeutics of ezetimibe (SCH 58235), a cholesterol-absorption inhibitor. *Clin. Ther.* 25, 2352-2387 (2003)).

TABLE 12



5

Analogue	X	Y	Rat BBM Ki (nM)	Rat NPC1L1 Ki (nM)	Human NPC1L1 Ki (nM)	Monkey BBM Ki (nM)
EZE-gluc <u>1</u>	OH ( <i>S</i> )	H	390	210	220	15
<u>4</u> *	OH ( <i>R</i> )	H	130,000	74,000	130,000	22,000
<u>3</u>	H	H	1,600	820	1,000	150
<u>6</u>	=O	H	33,000	23,000	14,000	3,300
<u>5</u>	OH ( <i>R</i> )	H	2,800	1,700	1,300	120
<u>7</u>	OH ( <i>S</i> )	OH	280	360	210	60

\* glucuronide of the enantiomer of ezetimibe has stereochemical configuration 3*S*, 4*R*

Conclusive evidence that NPC1L1 is the target of ezetimibe was provided by studies with tissues from NPC1L1 deficient mice. Enterocyte brush border membranes prepared from NPC1L1 deficient mice showed no detectable specific binding affinity for 1, whereas membranes from age-matched wild-type mice showed a high level of specific binding with a  $K_D = 12 \mu M$  (Figure 14).

For Figure 14A, enterocyte brush border membranes were prepared from NPC1L1 deficient male mice and same sex wild-type littermates, and tested for binding of 1. Conditions for binding were 5 mg/ml protein and 500 nM 1 in a volume of 20  $\mu l$  and in the presence of 0.03% sodium taurocholate and 0.05% digitonin. Membranes from wild type mice are on the left and from NPC1L1 deficient mice on the right. The bar graphs indicate total binding (left bar), nonspecific binding in the presence of 500  $\mu M$  cold ezetimibe glucuronide (middle bar), and specific (right bar) binding, respectively for each of wild type and NPC1L1 deficient mice, and error bars represent triplicate measurements. The graphs show that although specific binding is readily detectable in wild-type mice, it is absent in NPC1L1-deficient mice.

Figure 14B shows a plot demonstrating competition of unlabeled ezetimibe glucuronide against 1. Membranes from wild-type mice (upper curve) gave  $K_i = 12,000$  nM, while specific binding was virtually undetectable in membranes from the knockout animals (lower curve). Conditions were those described in Figure 14A.

The present studies involve a quantitative comparison of binding between recombinant proteins and brush border membranes. SR-BI (scavenger receptor type B1) was previously identified as a potential target using an expression cloning strategy employing ezetimibe binding to candidate proteins; this hypothesis was readily dismissed when neither cholesterol absorption nor ezetimibe activity were affected in SR-BI deficient mice (Altmann, S. W. *et al.* The identification of intestinal scavenger receptor class B, type 1 (SR-B1) by expression cloning and its role in cholesterol absorption. *Biochem. Biophys. Acta* 1580, 77-93 (2002)). The results show that ezetimibe binds to native intestinal membranes and cells expressing recombinant NPC1L1 with comparable affinity, and does not bind to membranes from NPC1L1 deficient mice, indicating a specific binding interaction between NPC1L1 and ezetimibe. Together with the previously published findings that mice deficient in NPC1L1 are defective in intestinal cholesterol uptake, and are no longer responsive to ezetimibe (Altmann, S. W. *et al.* Niemann-Pick C1 Like 1 protein is critical for



intestinal cholesterol absorption. *Science* 303, 1201-1204 (2004)), these data definitively establish NPC1L1 as the direct target of ezetimibe.

**Example 36: Effect of detergents on [<sup>3</sup>H]ezetimibe glucuronide binding.**

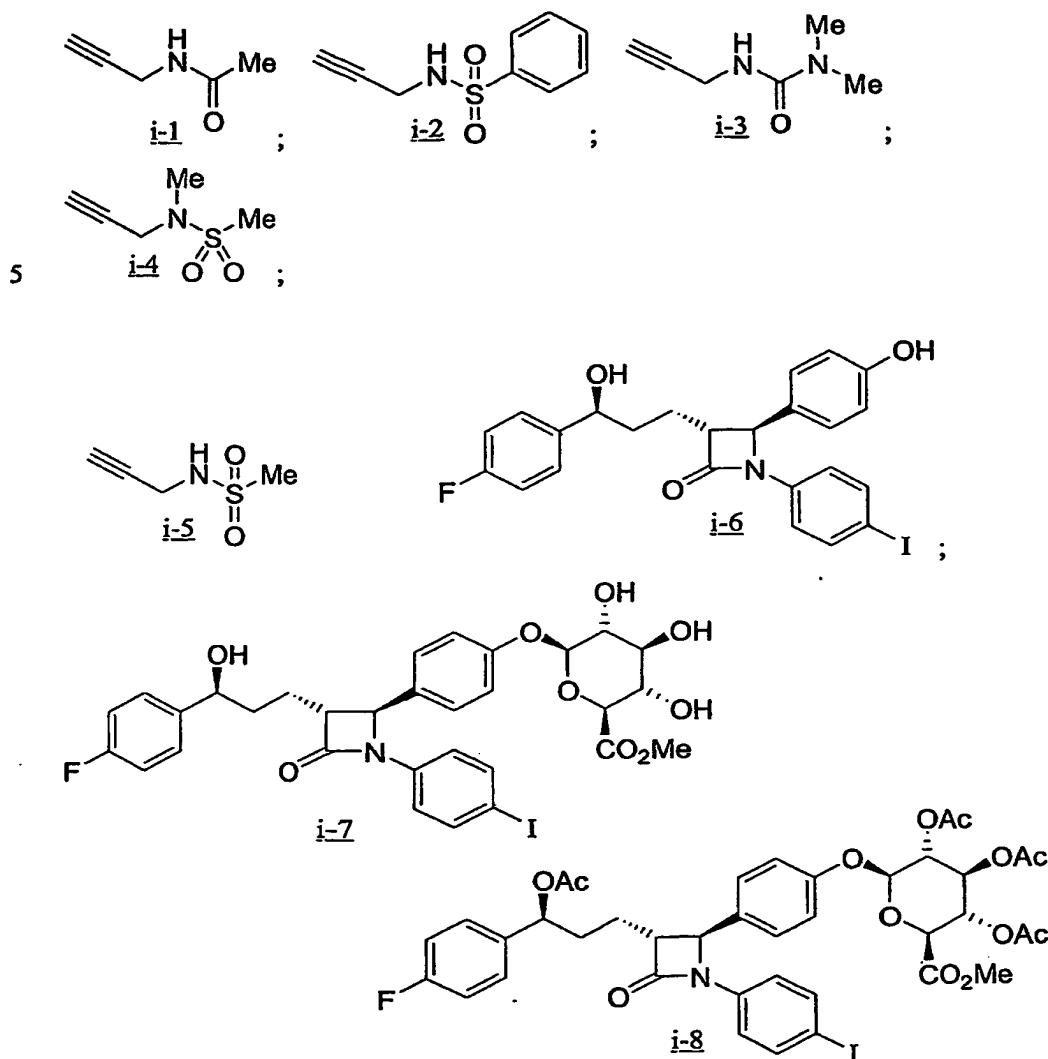
A practical aspect of work with the recombinant protein was that the number of binding sites in transfected NPC1L1-293 cell membranes initially appeared quite low. The influence of a combination of 0.03% taurocholate and 0.05% digitonin on specific binding to these and native enterocyte brush border membrane preparations is dramatic as illustrated in Figure 15.

Equal amounts (25 µg protein) of rat brush border membranes, membranes from HEK-293 cells transiently expressing recombinant rat and human NPC1L1, were incubated with 25 nM 1 in a final volume of 20 µl until equilibrium was achieved. The incubation conditions were buffer A with and without sodium taurocholate and digitonin to a final concentration of 0.03% and 0.05%, respectively. On the x-axis, "C" denotes controls in the absence of detergent, and "+det" the response in the presence of both detergents. The results are shown in 3 bar groupings; Total binding (left bar in each 3 bar group), nonspecific binding in the presence of 100 µM unlabeled ezetimibe glucuronide (middle bar in each 3 bar group), and specific binding (right bar in each 3 bar group) are shown.

**Example 37: Binding affinities of ezetimibe glucuronide and various analogues NPC1L1 in rat and rhesus monkey membranes.**

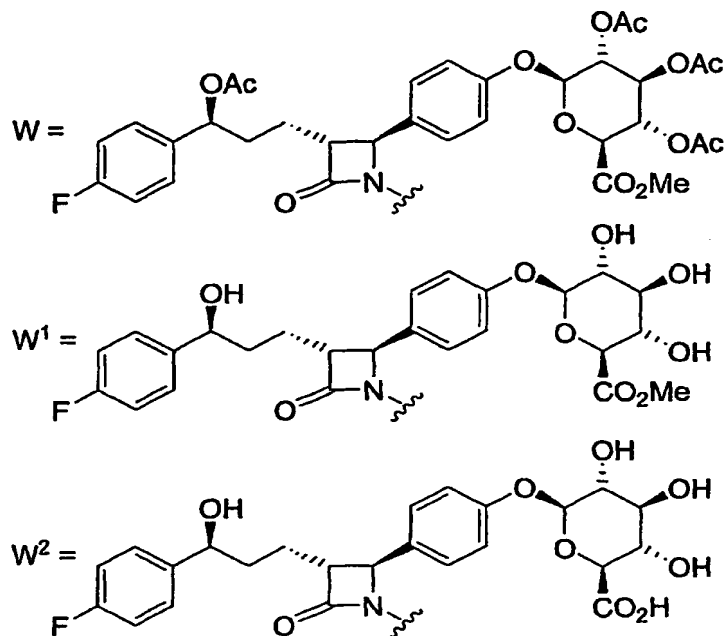
As determined from binding assay results using <sup>3</sup>H-ezetimibe glucuronide with rat brush border membrane, representative tested compounds of Formula II were determined to have IC<sub>50</sub>'s of about 13,000nM or lower, and particularly certain tested compounds had IC<sub>50</sub>'s of about 1900nM or lower, more particularly certain tested compounds had IC<sub>50</sub>'s of about 1000nM or lower, and most particularly certain tested compounds had IC<sub>50</sub>'s of less than 100nM. As determined from binding assay results using <sup>3</sup>H-ezetimibe glucuronide with rhesus brush border membrane, representative tested compounds of Formula II were determined to have IC<sub>50</sub>'s of about 4200nM or lower, and particularly certain tested compounds had IC<sub>50</sub>'s of about 165nM or lower, more particularly certain tested compounds had IC<sub>50</sub>'s of less than 100nM, and most particularly certain tested compounds had IC<sub>50</sub>'s of less than 50nM.

The designations below are used in the Examples that follow for certain repetitively used intermediates:



The compounds (3*R*,4*S*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-1-(4-iodophenyl)azetidin-2-one (i-6) and 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-iodophenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (i-7) were prepared according to Burnett, D. S.; Caplen, M. A.; Domalski, M. S.; Browne, M. E.; Davis, H. R. Jr.; Clader, J. W. *Bioorg. Med. Chem. Lett.* (2002), 12, 311. Compound i-8 is the hydroxy-protected analog of i-7, where the protecting group is acyl.

The following definitions are also used in the Examples that follow:



### EXAMPLE 38

#### 5 Preparation of *N*-prop-2-yn-1-ylacetamide (i-1)

Acetyl chloride (0.52 mL, 7.3 mmol) was added to a stirred solution of propargylamine (0.5 mL, 7.3 mmol) and dimethylaminopyridine (18 mg, 0.14 mmol) in pyridine (2.5 mL) at 0°C, and the resulting mixture was allowed to warm to ambient temperature. After approximately 15 h, the reaction mixture was diluted with ethyl acetate and washed successively with 1N HCl and brine. The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and concentrated *in vacuo* to afford the title compound (i-1), which was used without further purification.

### EXAMPLE 39

#### 15 Preparation of *N*-prop-2-yn-1-ylbenzenesulfonamide (i-2)

Benzene sulfonyl chloride (1.16 mL, 9.1 mmol) was added to stirred solution of propargylamine (0.62 mL, 9.1 mmol) and dimethylaminopyridine (22 mg, 0.18 mmol) in pyridine (5 mL) at room temperature. The resulting solution was aged

at ambient temperature for approximately 15 h. The reaction mixture was diluted with ethyl acetate and washed successively with 1N HCl and brine. The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and concentrated *in vacuo* to furnish the title compound (i-2), which was used without further purification.

5

## EXAMPLE 40

Preparation of *N,N*-Dimethyl-*N'*-prop-2-yn-1-ylurea (i-3)

Dimethyl carbamylchloride (0.84 mL, 9.1 mmol) was added to a stirred solution of propargylamine (0.62 mL, 9.1 mmol) and dimethylaminopyridine (22 mg, 0.18 mmol) in pyridine (5 mL) at room temperature. The resulting  
10 suspension was stirred at ambient temperature for approximately 15 h. The reaction mixture was diluted with ethyl acetate and washed successively with 1N HCl and brine. The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and concentrated *in vacuo* to afford the title compound (i-3), which was used without further purification.

## EXAMPLE 41

15 Preparation of *N*-Methyl-*N*-prop-2-yn-1-ylmethanesulfonamide (i-4)

Methansulfonylchloride (1.12 mL, 14.5 mmol) was added to a stirred solution of *N*-methylpropargylamine (1.22 mL, 14.5 mmol) and dimethylaminopyridine (35 mg, 0.30 mmol) in pyridine (10 mL) at room temperature. After aging for approximately 15 h, the reaction mixture was poured into ethyl acetate  
20 and washed successively with 1N HCl and brine. The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and concentrated *in vacuo*, to afford the title compound (i-4), which was used without further purification.

## EXAMPLE 42

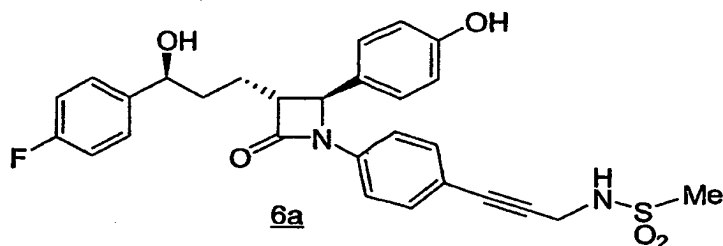
Preparation of *N*-prop-2-yn-1-ylmethanesulfonamide (i-5)

25 Methansulfonylchloride (1.40 mL, 18.1 mmol) was added dropwise to a stirred solution of propargylamine (1.00 g, 18.1 mmol) and dimethylaminopyridine (44.0 mg, 0.36 mmol) in pyridine (10 mL) at 0 °C. After aging for approximately 15 h, the reaction mixture was poured into 1N HCl and extracted twice with ethyl acetate. The combined organic extracts were washed with saturated aqueous sodium  
30 bicarbonate, brine, dried (MgSO<sub>4</sub>), filtered and concentrated *in vacuo*, to afford the

title compound i-5. Crude i-5 crystallized on standing and was used without further purification.

#### EXAMPLE 43

Preparation of *N*-(3-{4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-2-(4-hydroxyphenyl)-4-oxoazetidin-1-yl]phenyl}prop-2-yn-1-yl)methanesulfonamide (Compound 6a)

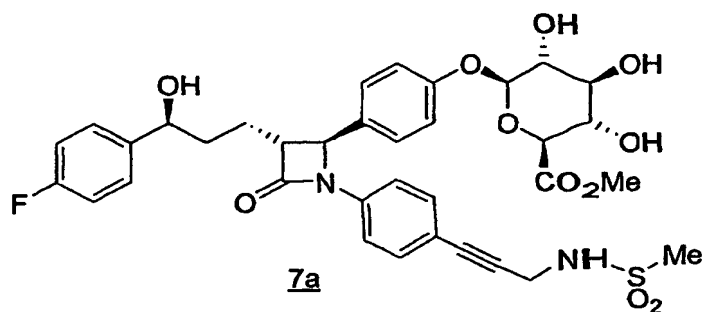


Triethylamine (7 equivalents) is added to a solution of (3*R*,4*S*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-1-(4-iodophenyl)azetidin-2-one (*i*-6) (1.00 equivalent), *N*-prop-2-yn-1-ylmethanesulfonamide (*i*-5) (1.50 equivalents), tetrakis(triphenylphosphine) palladium(0) (0.15 equivalents) and copper(I) iodide (0.30 equivalents) in DMF (0.1 M concentration with respect to final product) under a nitrogen atmosphere and the resulting solution aged at room temperature. After completion of reaction, the volatiles are evaporated in vacuo and the crude residue can be purified by flash chromatography on silica gel to afford the title compound.

## EXAMPLE 44

Step A: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (Compound 7a)

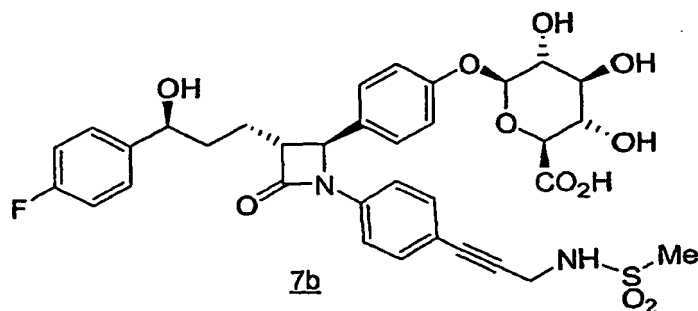
5



Triethylamine (0.07 mL, 0.502 mmol) was added to a stirred solution of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-iodophenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (**i-7**) (0.050 g, 0.071 mmol), *N*-prop-2-yn-1-ylmethanesulfonamide (**i-5**) (0.014 g, 0.105 mmol), tetrakis(triphenylphosphine) palladium(0) (0.012 g, 0.010 mmol) and copper iodide (0.005 g, 0.026 mmol) in DMF (0.5 mL) under a nitrogen atmosphere and the resulting solution aged at room temperature for 18 h. The volatiles were evaporated in vacuo and the crude residue purified by flash chromatography on silica gel (gradient elution; 0-25% methanol/methylene chloride as eluent) to afford the title compound; *m/z* (ES) 713 (MH<sup>+</sup>), 505.

15

Step B: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid (Compound 7b, also referred to herein as compound 2)



5

A solution of compound **7a** in methanol/water/triethylamine (1:7:2; 1 mL) was stirred at room temperature for approximately 1.5 h. The volatiles were evaporated *in vacuo* and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-65% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (**7b**); *m/z* (ES) 699 ( $MH^+$ ), 505; HRMS (ES) *m/z* calcd for  $C_{34}H_{36}FN_2O_{11}S$  ( $MH^+$ ) 699.2024, found 699.2016.

10

#### EXAMPLE 45 (COMPOUNDS 6B TO 6G AND 7C TO 7N)

The following compounds of Formula IIa have been prepared (as indicated by MS data provided) or can be prepared using the general synthetic procedures described in Example 43 (shown in Table 13) or Example 44 (shown in Table 14).

15

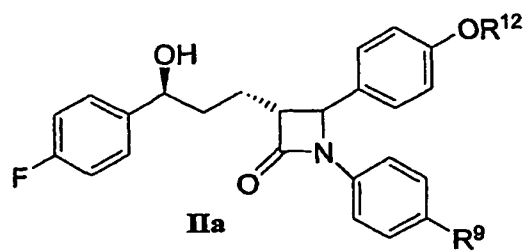


TABLE 13

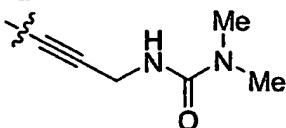
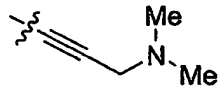
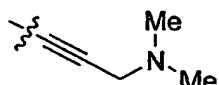
Compound	R <sup>12</sup>	R <sup>9</sup>
6b)	H	
6c)	H	
6d)	H	
6e)	H	
6f)	H	
6g)	H	



TABLE 14

Compound	R12	R9	<i>m/z</i> (ES)	HRMS <i>m/z</i> (ES)
7c)	methyl ester glucuronide		658 (MNa <sup>+</sup> )	
7d)	glucuronide		621 (MH <sup>+</sup> )	(MH <sup>+</sup> ) Calcd 621.2249 Found 621.2223
7e)	methyl ester glucuronide		677 (MH <sup>+</sup> )	
7f)	glucuronide		663 (MH <sup>+</sup> )	(MH <sup>+</sup> ) Calcd 663.2354 Found 663.2331
7g)	methyl ester glucuronide		749 (MNa <sup>+</sup> )	
7h)	glucuronide		735 (MNa <sup>+</sup> )	(MH <sup>+</sup> ) Calcd 713.2180 Found 713.2170
7i)	methyl ester glucuronide		797 (MNa <sup>+</sup> )	
7j)	glucuronide		783 (MNa <sup>+</sup> )	(MH <sup>+</sup> ) Calcd 761.2180 Found 761.2193
7k)	methyl ester glucuronide		706 (MH <sup>+</sup> )	

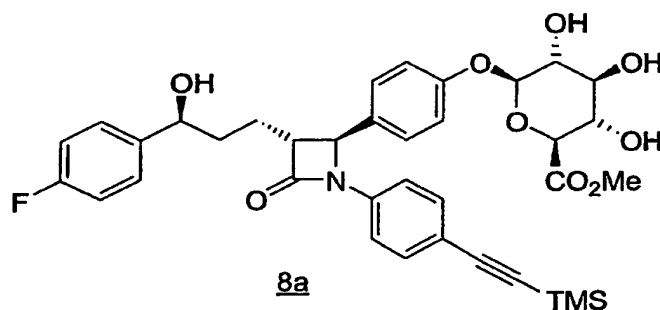
TABLE 14

Compound	R <sup>12</sup>	R <sup>9</sup>	m/z (ES)	HRMS m/z (ES)
7l)	glucuronide		692 (MH <sup>+</sup> )	(MH <sup>+</sup> ) Calcd 692.2620 Found 692.2618
7m)	methyl ester glucuronide		663 (MH <sup>+</sup> )	
7n)	glucuronide		649 (MH <sup>+</sup> )	(MH <sup>+</sup> ) Calcd 649.2562 Found 649.2532

## EXAMPLE 46

Step A: Preparation of 4-((2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-oxo-1-[4-[(trimethylsilyl)ethynyl]phenyl]azetidin-2-yl)phenyl methyl β-D-glucopyranosiduronate (8a)

5

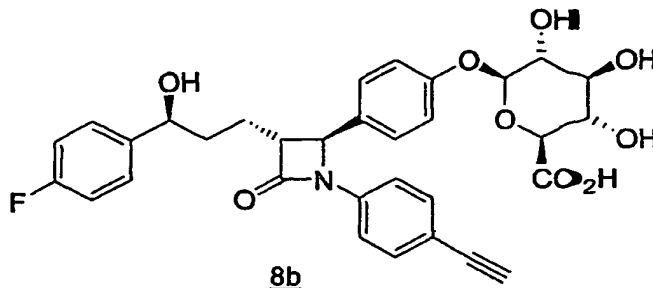


Triethylamine (69.0 μL, 0.495 mmol) was added to a stirred solution of **i-7** (50.0 mg, 0.071 mmol), trimethylsilylacetylene (12.0 μL, 0.085 mmol), tetrakis(triphenylphosphine) palladium(0) (13.0 mg, 0.011 mmol) and copper iodide (5.10 mg, 0.028 mmol) in DMF (0.5 mL) under a nitrogen atmosphere and the resulting solution aged at room temperature for 18 h. The volatiles were evaporated in vacuo and the crude residue purified by flash chromatography on silica gel

10

(gradient elution; 0-25% methanol/methylene chloride as eluent) to afford the title compound (**8a**);  $m/z$  (ES) 660 (M-OH)<sup>+</sup>, 470.

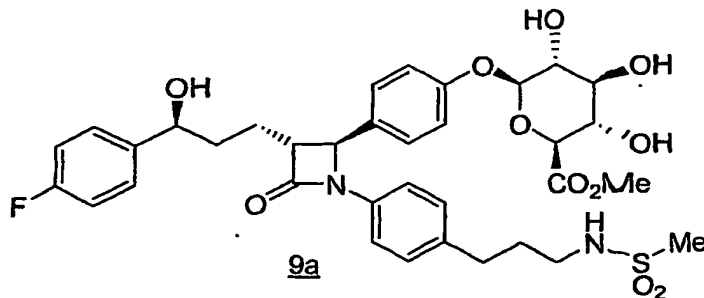
Step B: Preparation of 4-[(2*S*,3*R*)-1-(4-ethynylphenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid (**8b**)



A solution of **8a** in methanol/water/triethylamine (0.25 mL:1.10 mL:0.40 mL) was stirred at room temperature for approximately 6 h. The volatiles were evaporated *in vacuo* and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-65% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (**8b**);  $m/z$  (ES) 574 (M-OH)<sup>+</sup>, 398; HRMS (ES)  $m/z$  calc'd for C<sub>32</sub>H<sub>31</sub>FNO<sub>9</sub> (MH<sup>+</sup>) 592.1983, found 592.1985.

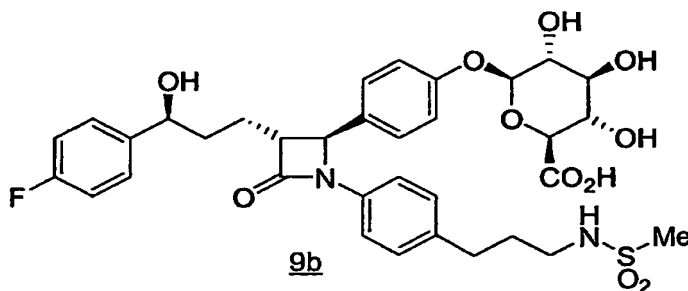
#### EXAMPLE 47

Step A: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]propyl}phenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (**9a**)



A mixture of **7a** (40.0 mg, 0.056 mmol) and palladium (~8 mg of 10 wt. % (dry basis) on activated carbon) in methanol (2 mL) was hydrogenated at atmospheric pressure for approximately 1 h. The reaction mixture was filtered through a short plug of celite, eluting copiously with methanol, and the filtrate  
 5 evaporated *in vacuo* to afford the title compound (**9a**); *m/z* (ES) 509 (M-sugar-OH)<sup>+</sup>.

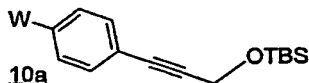
Step B: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]propyl}phenyl)-4-oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid (**9b**)



A solution of **9a** in methanol/water/triethylamine (1:7:2, 1 mL) was stirred at room temperature for approximately 1 h. The volatiles were evaporated *in vacuo* and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-65% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (**9b**); *m/z*  
 10 (ES) 735 (M+Na)<sup>+</sup>, 685 (M-OH)<sup>+</sup>, 509 (M-sugar-OH)<sup>+</sup>; HRMS (ES) *m/z* calc'd for C<sub>34</sub>H<sub>39</sub>FN<sub>2</sub>O<sub>11</sub>S (MH<sup>+</sup>) 703.2337, found 703.2337.

#### EXAMPLE 48

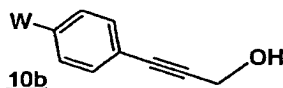
Step A: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-acetoxy)-3-(4-fluorophenyl)propyl]-1-[4-(3-{[*tert*-butyl(dimethylsilyl)oxy]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (**10a**)



Triethylamine (170 μL, 1.25 mmol) was added to a solution of **i-8** (156 mg, 0.178 mmol), *tert*-butyldimethyl(2-propynyloxy)silane (43.0 μL, 0.214 mmol),

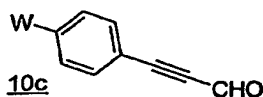
dichlorobis(triphenylphosphine)palladium(II) (12.0 mg, 0.018 mmol) and copper iodide (7.00 mg, 0.036 mmol) in DMF (1.3 mL) under a nitrogen atmosphere and the resulting solution aged at room temperature for approximately 20 h. The reaction mixture was poured into saturated aqueous sodium bicarbonate and extracted twice with diethyl ether. The combined organic extracts were washed with water, brine, dried (MgSO<sub>4</sub>), filtered and the filtrate concentrated *in vacuo*. Purification of the crude residue by flash chromatography on silica gel (gradient elution; 15-40% ethyl acetate/hexanes as eluent) afforded the title compound 10a.

Step B: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-[4-(3-hydroxyprop-1-yn-1-yl)phenyl]-4-oxoazetidin-2-yl]phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (10b)



Tetrabutylammonium fluoride hydrate (39.0 mg, 0.148 mmol) was added to 10a (136 mg, 0.148 mmol) in tetrahydrofuran (1.5 mL), and the resulting solution aged at room temperature for 1 h. The reaction mixture was poured into saturated aqueous ammonium chloride and extracted twice with ether. The combined organic extracts were washed with saturated sodium bicarbonate, brine, dried (MgSO<sub>4</sub>), filtered and concentrated *in vacuo*. Purification of the crude residue by flash chromatography on silica gel (50% ethyl acetate/hexanes) afforded the title compound 10b.

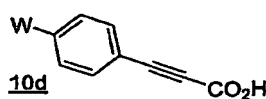
Step C: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-4-oxo-1-[4-(3-oxoprop-1-yn-1-yl)phenyl]azetidin-2-yl]phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (10c)



Dess-Martin periodinane (33.0 mg, 0.077 mmol) was added to a solution of 10b (62.0 mg, 0.077 mmol) and pyridine (31.0 μL, 0.386 mmol) in dichloromethane (1 mL) at room temperature. After 30 min, the reaction mixture was

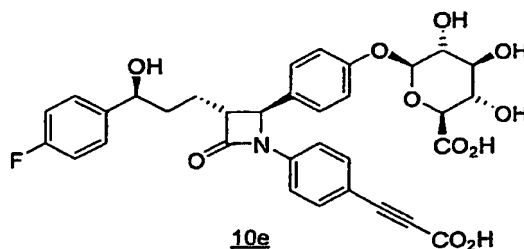
poured into saturated aqueous sodium bicarbonate, and extracted twice with ethyl acetate. The combined organic extracts were washed with water, brine, dried ( $\text{MgSO}_4$ ), filtered and concentrated *in vacuo*. Purification of the crude residue by flash chromatography on silica gel (gradient elution; 20-40% ethyl acetate/hexanes) afforded the title compound **10c**.

Step D: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-[4-(carboxyethynyl)phenyl]-4-oxoazetidin-2-yl]phenyl methyl 2,3,4-tri-*O*-acetyl- $\beta$ -D-glucopyranosiduronate (**10d**)



An aqueous solution (0.1 mL) of sodium dihydrogenphosphate (9.00 mg, 0.065 mmol) and sodium chlorite (5.00 mg, 0.055 mmol) was added to a solution of **10c** (37.0 mg, 0.046 mmol) in *tert*-butyl alcohol (0.4 mL), dioxane (0.2 mL) and isobutylene (~0.1 mL) at room temperature. After 1.5 h, the reaction mixture was concentrated *in vacuo* and the crude residue triturated repeatedly with ethyl acetate. The organic washings were dried ( $\text{Na}_2\text{SO}_4$ ), filtered and concentrated *in vacuo* to afford the title compound **10d**.

Step E: Preparation of 4-[(2*S*,3*R*)-1-[4-(carboxyethynyl)phenyl]-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-oxoazetidin-2-yl]phenyl  $\beta$ -D-glucopyranosiduronic acid (**10e**)

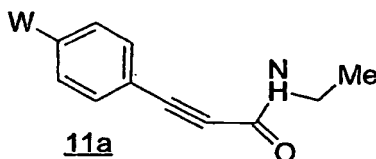


A solution of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-[4-(carboxyethynyl)phenyl]-4-oxoazetidin-2-yl]phenyl methyl 2,3,4-tri-*O*-acetyl- $\beta$ -D-glucopyranosiduronate (**10d**) and sodium cyanide (~1mg, 0.020 mmol) in methanol (3mL) was heated to 45°C. After 22 h, the reaction mixture was concentrated under reduced pressure and dissolved in methanol/water/triethylamine (1:7:2, 1 mL). After stirring at room temperature for

approximately 1 h, the volatiles were evaporated *in vacuo* and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-60% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (10e), *m/z* (ES) 442.0 (M-sugar-OH)<sup>+</sup>, 618.0 (M-OH)<sup>+</sup>; HRMS (ES) *m/z* calcd. for C<sub>33</sub>H<sub>31</sub>FNO<sub>11</sub> (MH<sup>+</sup>) 636.1881, found 636.1889

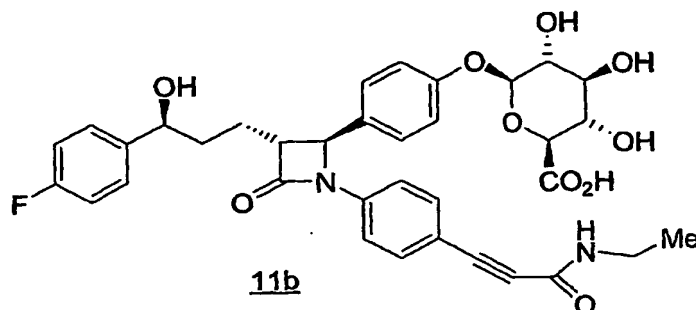
## EXAMPLE 49

Step A: Preparation of 4-((2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-{4-(3-(ethylamino)-3-oxoprop-1-yn-1-yl]phenyl}-4-oxoazetidin-2-yl)phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (11a)



A 1M solution of ethylamine hydrochloride and diisopropylethylamine in DMF (40.0 μL, 0.40 mmol) was added to 4-((2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-{4-(carboxyethynyl)phenyl}-4-oxoazetidin-2-yl}phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (10d) (27.0 mg, 0.033 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (19.0 mg, 0.099 mmol) and 1-hydroxybenzotriazole (8.00 mg, 0.059 mmol) in DMF (0.25 mL). After 4.5 h, the reaction mixture was poured into ethyl acetate and washed successively with water and brine. The organic layer was dried, filtered and concentrated under reduced pressure. Purification of the crude residue by flash chromatography on silica gel (gradient elution; 50-60% ethyl acetate/hexanes) afforded the title compound 11a.

Step B: Preparation of 4-((2*S*,3*R*)-1-{4-[3-(ethylamino)-3-oxoprop-1-yn-1-yl]phenyl}-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-oxoazetidin-2-yl}phenyl β-D-glucopyranosiduronic acid (11b)



5 A solution of 4-((2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-{4-(3-(ethylamino)-3-oxoprop-1-yn-1-yl]phenyl}-4-oxoazetidin-2-yl)phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (11a) (22.0 mg, 0.026 mmol) and sodium cyanide (~1mg, 0.020 mmol) in methanol (3mL) was heated to 45°C. After 18 h, the reaction mixture was concentrated under reduced  
10 pressure and dissolved in methanol/water/triethylamine (1:3:1, 2.5 mL). After stirring at room temperature for approximately 1 h, the volatiles were evaporated *in vacuo*, and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-60% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (11b) *m/z* (ES) 663.0 (M+H)<sup>+</sup>; HRMS (ES) *m/z* calcd. for C<sub>35</sub>H<sub>36</sub>FN<sub>2</sub>O<sub>10</sub> (MH<sup>+</sup>) 663.2354, found 663.2341.  
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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in  
20 addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Patents, patent applications, publications, product descriptions, Genbank Accession Numbers and protocols are cited throughout this application, the  
25 disclosures of which are incorporated herein by reference in their entireties for all purposes.



### CLAIMS

1. A method for identifying a ligand of NPC1L1 comprising:  
contacting human NPC1L1 with a detectably labeled substituted 2-azetidinone glucuronide and a candidate compound; and  
determining whether said candidate compound binds to human NPC1L1;  
wherein binding of said candidate compound to human NPC1L1 modulates binding of said detectably labeled substituted 2-azetidinone glucuronide to human NPC1L1, wherein the detectably labeled substituted 2-azetidinone glucuronide has a binding affinity  $K_D$  value for human NPC1L1 that is 200nM or lower, and wherein said modulation indicates that the candidate compound is a ligand that binds to human NPC1L1.
2. The method of claim 1, wherein the  $K_D$  value is 100nM or lower.
3. The method of claim 1, wherein the  $K_D$  value is 50nM or lower.
4. The method of claim 1, wherein the  $K_D$  value is 20nM or lower.
5. The method of claim 1, wherein the  $K_D$  value is 10nM or lower.
6. The method of claim 1, wherein the substituted 2-azetidinone-glucuronide is selected from the group consisting of a compound of Formula I and a compound of Formula II.
7. The method of claim 6, wherein the substituted 2-azetidinone-glucuronide comprises a detectable label from the group consisting of  $^{35}\text{S}$  and  $^{125}\text{I}$ .
8. The method of claim 7, wherein the detectable label is  $^{35}\text{S}$ .
9. The method of claim 6, wherein the substituted 2-azetidinone-glucuronide is a compound of Formula II, wherein  $\text{R}^9$  comprises an  $-\text{SO}_2-$  group.
10. The method of claim 9, wherein the substituted 2-azetidinone-glucuronide of Formula II is labeled with  $^{35}\text{S}$ .

11. A method for identifying a ligand of NPC1L1 comprising:
- contacting human NPC1L1 with a detectably labeled substituted 2-azetidinone glucuronide of Formula II and a candidate compound; and
- determining whether said candidate compound binds to human NPC1L1;
- wherein binding of said candidate compound to human NPC1L1 modulates binding of said detectably labeled substituted 2-azetidinone glucuronide of Formula II to human NPC1L1, and wherein said modulation indicates that the candidate compound is a ligand that binds to human NPC1L1.
12. The method of claim 11, wherein R<sup>9</sup> of the detectably labeled substituted 2-azetidinone glucuronide of Formula II comprises an -SO<sub>2</sub>- group.
13. The method of claim 11, wherein the detectably labeled substituted 2-azetidinone glucuronide of Formula II is labeled with <sup>35</sup>S.
14. The method of claim 11, wherein the detectably labeled substituted 2-azetidinone glucuronide of Formula II has a binding affinity K<sub>D</sub> value for human NPC1L1 that is 200nM or lower.
15. The method of claim 14, wherein the K<sub>D</sub> value is 100nM or lower.
16. The method of claim 14, wherein the K<sub>D</sub> value is 50nM or lower.
17. The method of claim 14, wherein the K<sub>D</sub> value is 20nM or lower.
18. The method of claim 14, wherein the K<sub>D</sub> value is 10nM or lower.
19. The method of claim 1 wherein the detectably labeled substituted 2-azetidinone glucuronide is labeled with <sup>35</sup>S.
20. The method of claim 1 wherein the detectably labeled substituted 2-azetidinone glucuronide is <sup>35</sup>S-labeled compound 2.

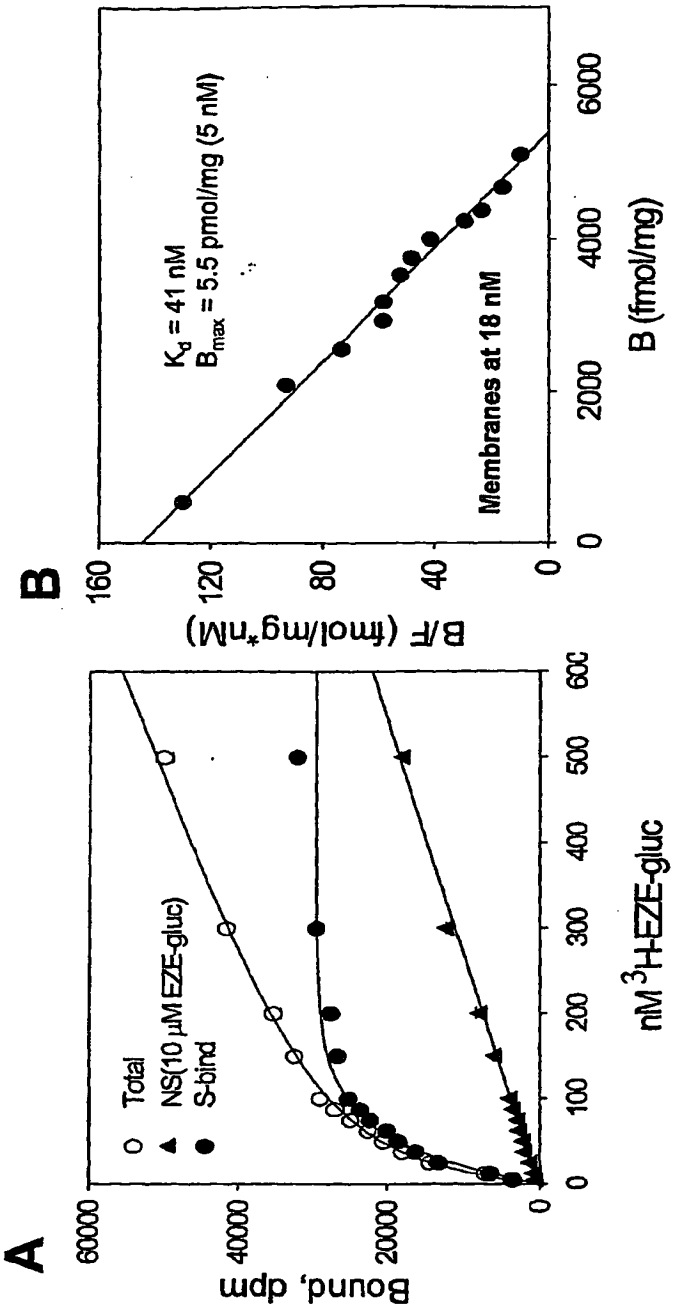


Figure 1. Equilibrium binding of EZE-glucuronide to rhesus BBMVs

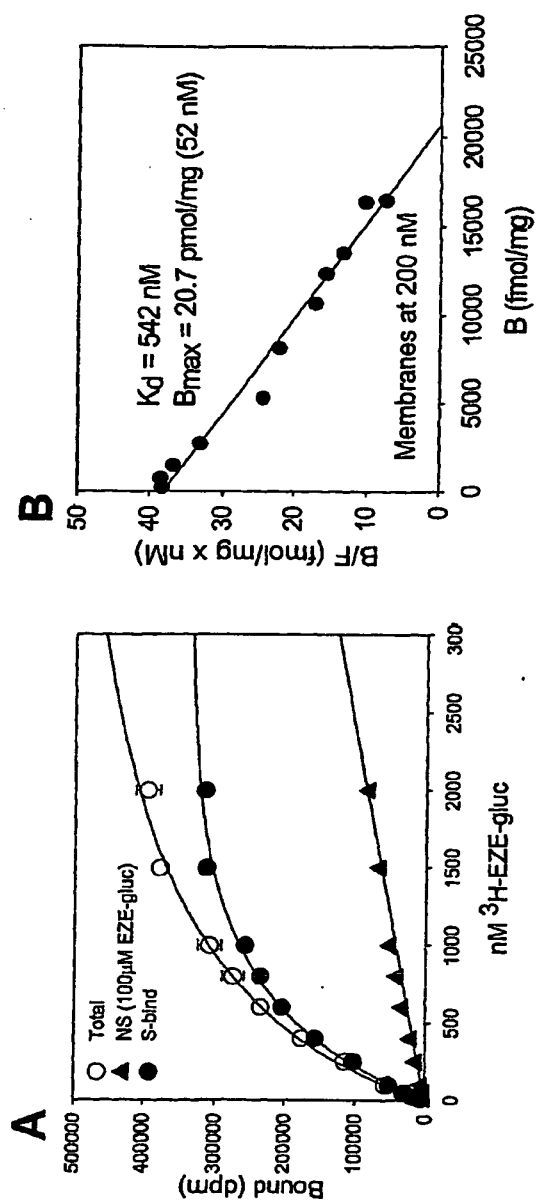
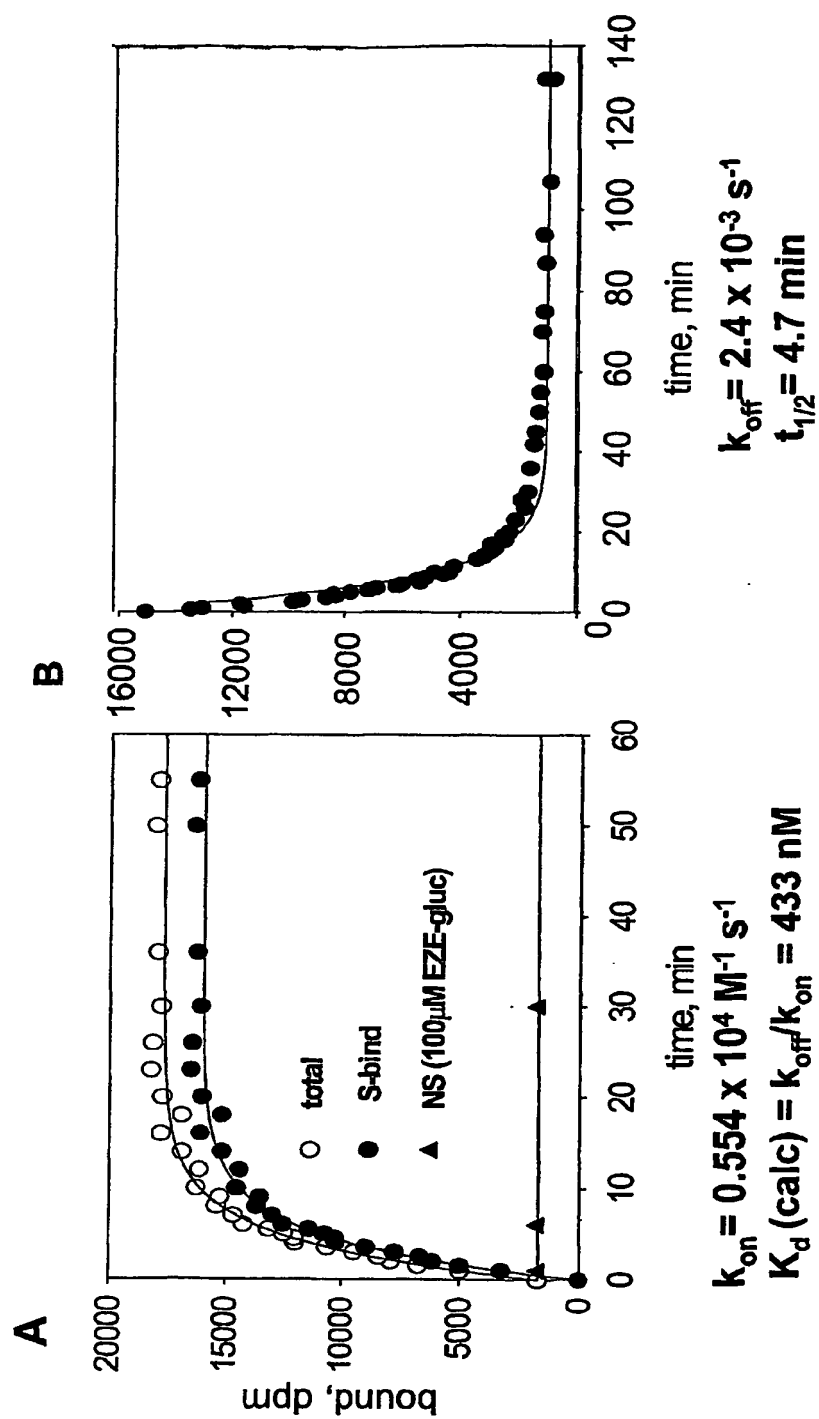
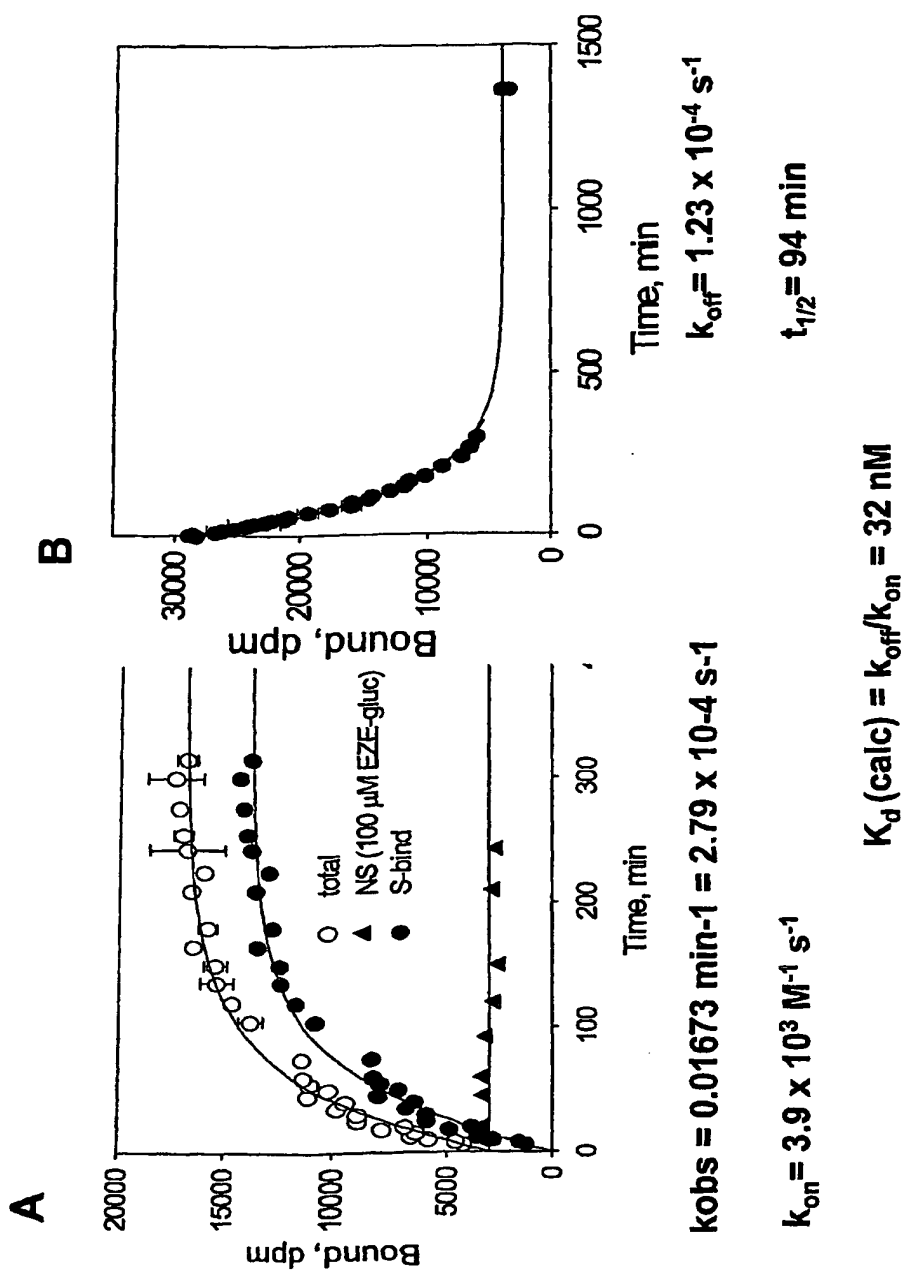


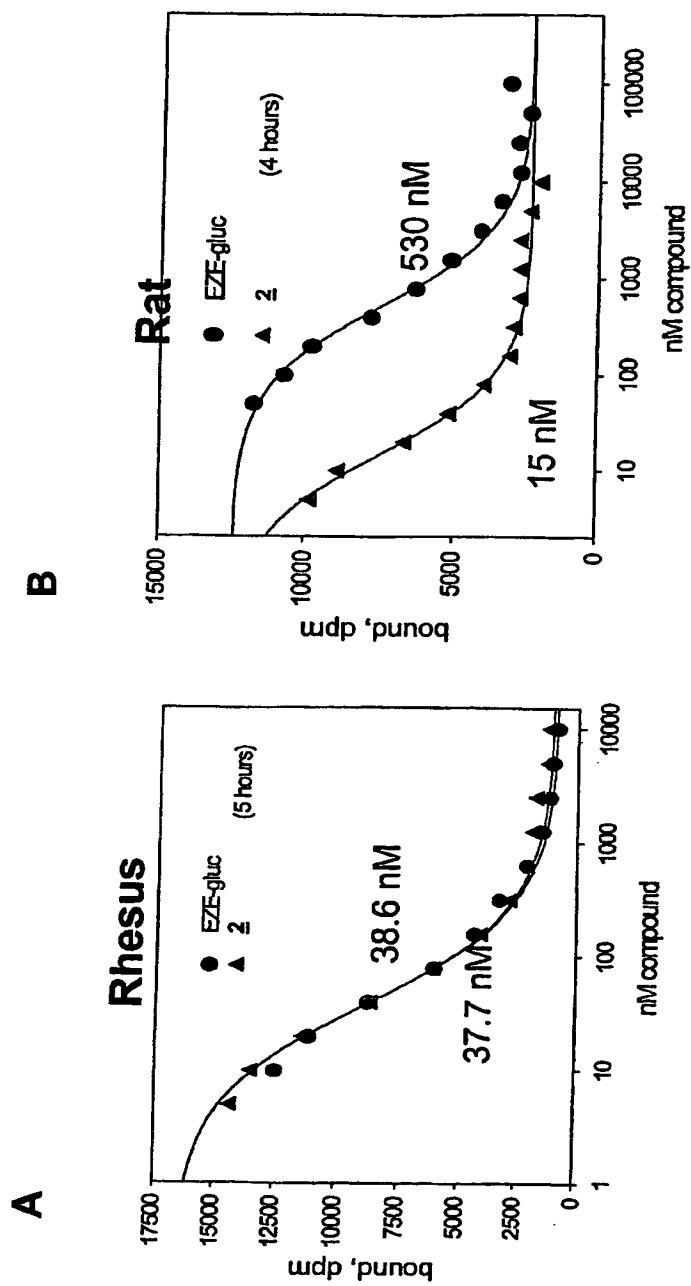
Figure 2. Equilibrium binding of EZE-glucuronide to rat BBMVs.



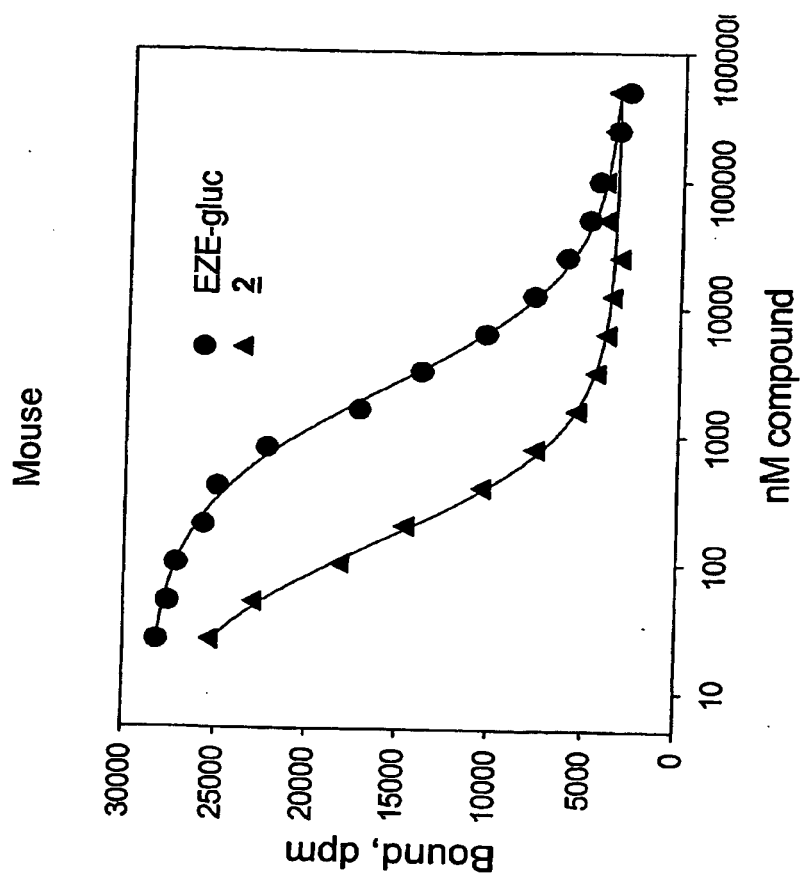
**Figure 3. Association and dissociation kinetics of  $^3\text{H}$ -EZE-glucuronide in rat BBMV.**



**Figure 4. Association and Dissociation kinetics of  $^3\text{H}$ -EZE-glucuronide in rhesus BBMV.**



**Figure 5. Displacement of 3H-EZE-glucuronide by EZE-glucuronide and compound 2 in rhesus and rat BMV.**

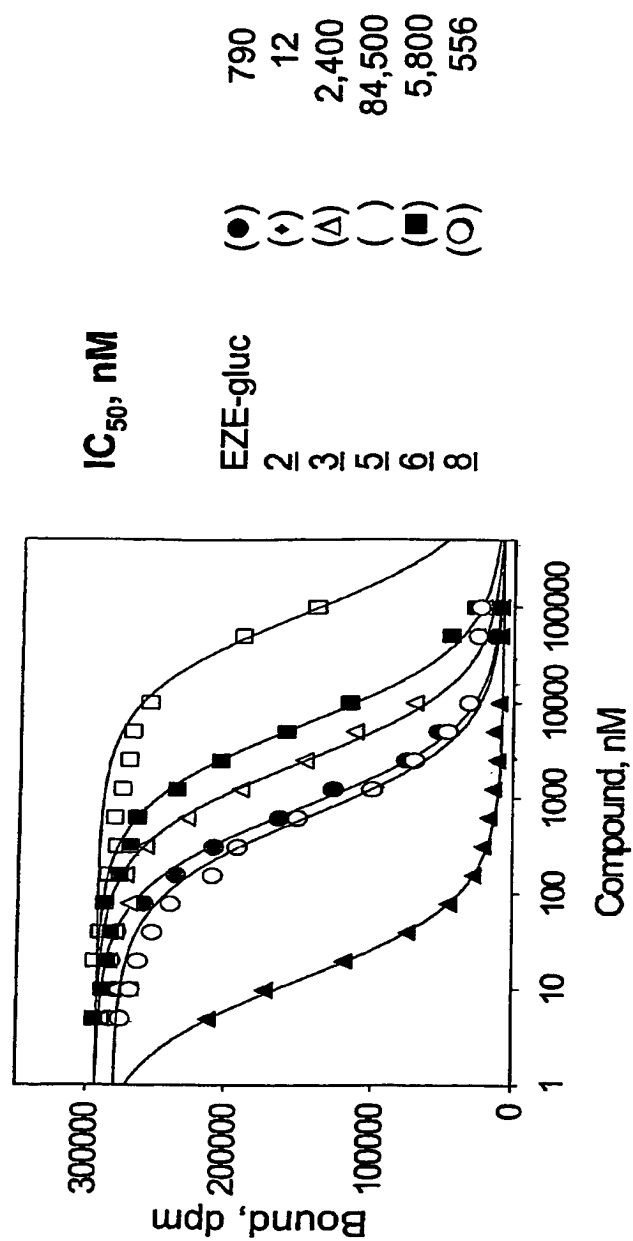


**Figure 6. Displacement of <sup>35</sup>S-labeled compound 2 by EZE-glucuronide and compound 2 in mouse BMV.**





Figure 7. Intestinal distribution of ezetimibe binding sites.



**Figure 8. Displacement of  $^{35}\text{S}$ -labeled compound **2** by EZE-glucuronide and analogs in transfected CHO cells expressing rat NPC1L1**

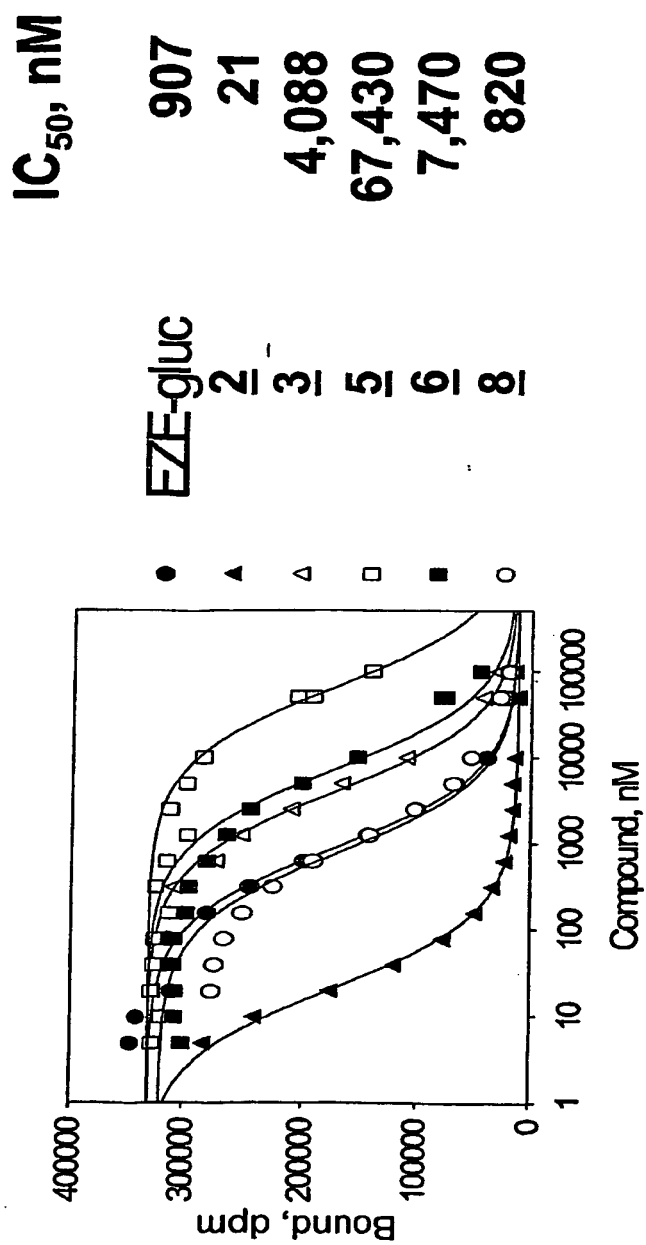
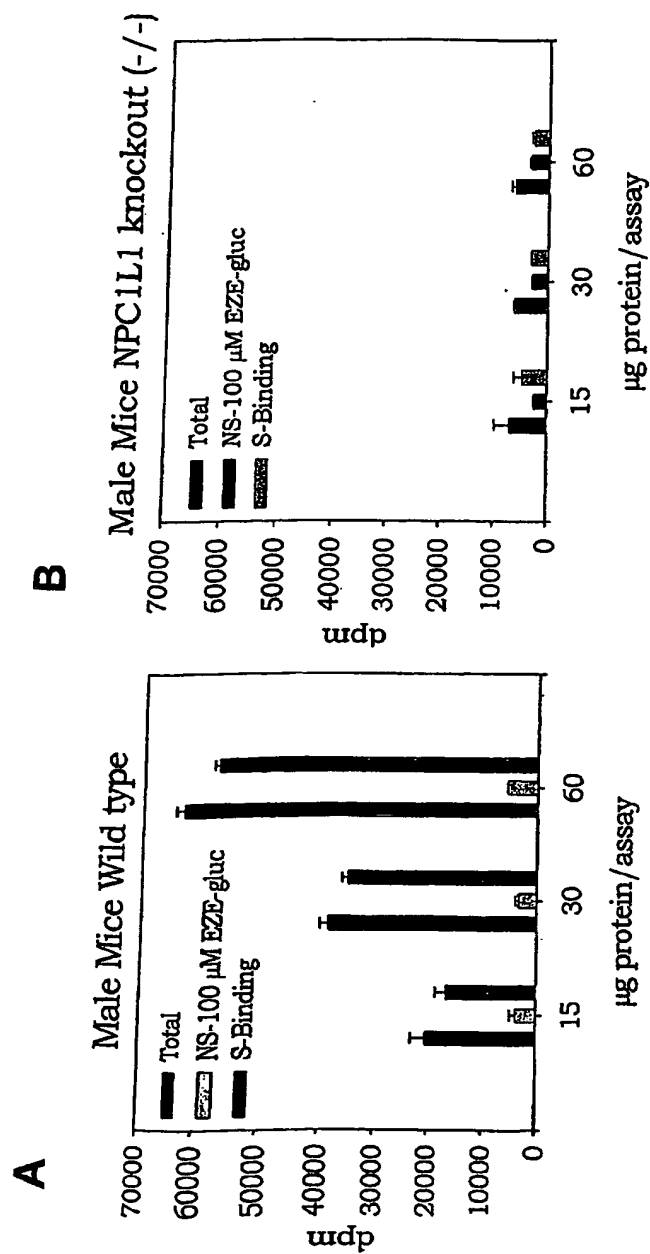


Figure 9. Displacement of <sup>35</sup>S-labeled compound 2 by EZE-glucuronide and analogs in transfected CHO cells expressing human NPC1L1



**Figure 10.**  $^{35}\text{S}$ -labeled compound 2 binding with brush border membranes from intestinal mucosal scrapings of male wild type (A) and NPC1L1 knockout (-/-) mice (B).

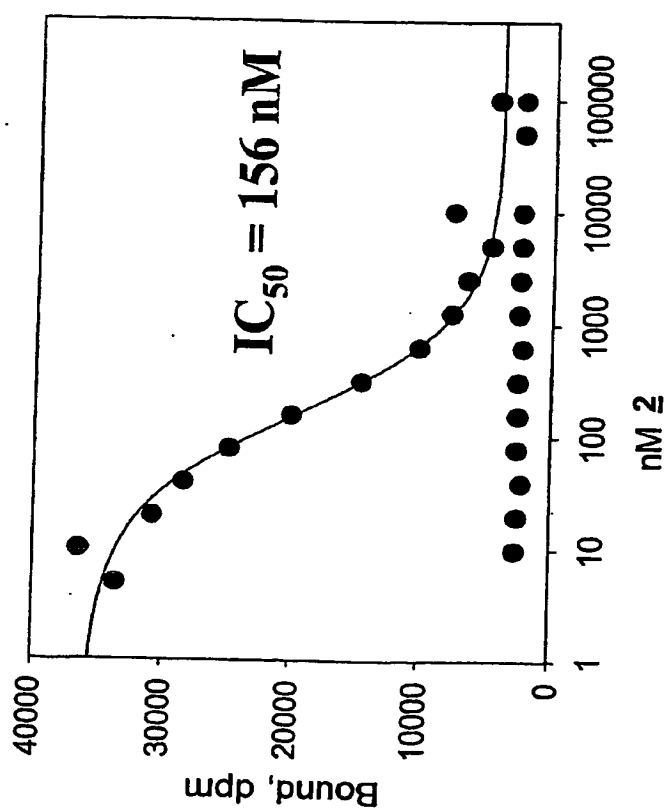


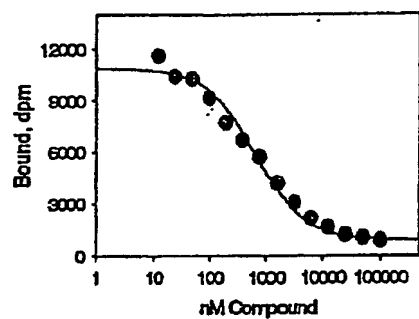
Figure 11. Displacement of  $^{35}\text{S}$ -labeled compound 2 by compound 2 in mouse wild type and knockout mouse NPC1L1 (-/-) BBMVs.

FIGURE 12

## Competition

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A



B

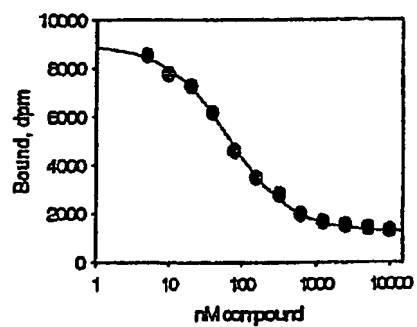
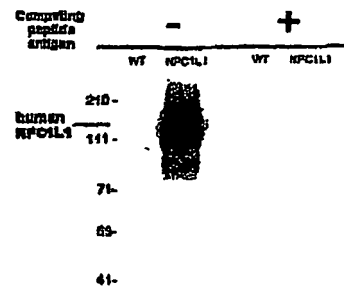


FIGURE 13

Panel 1



Panel 2

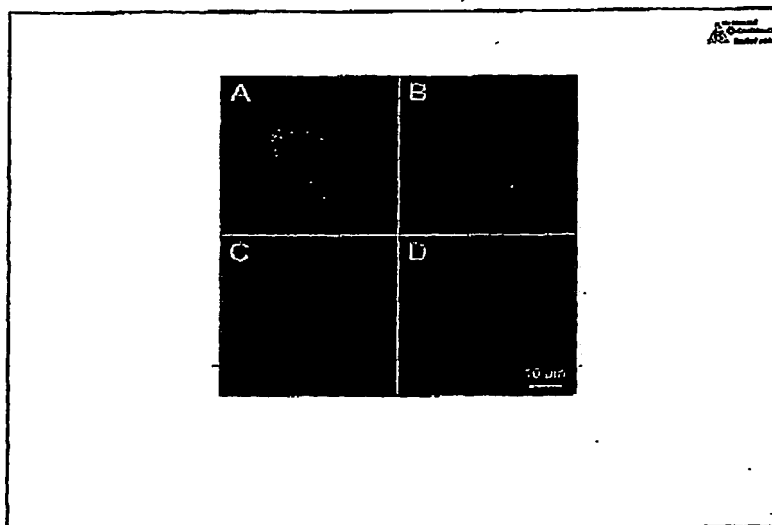


FIGURE 14

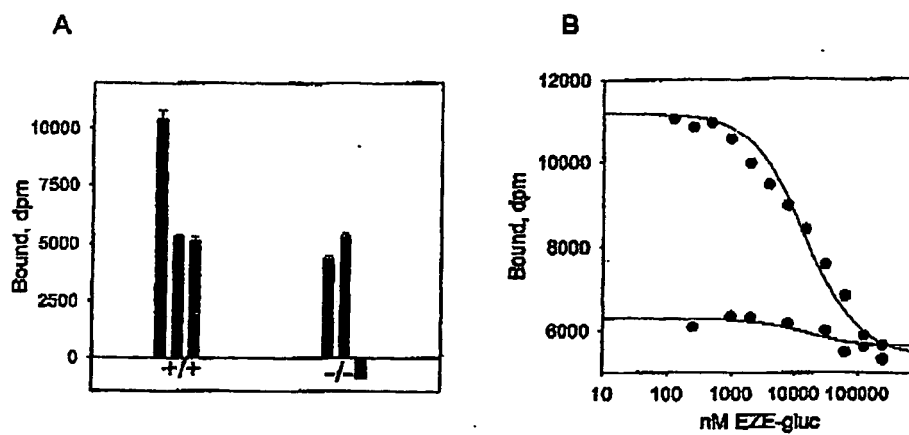
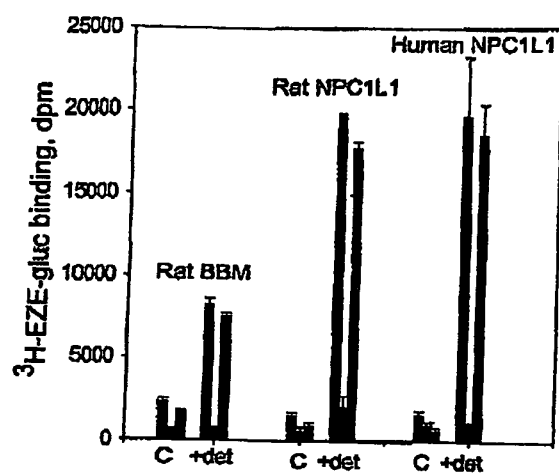




FIGURE 15



## SEQUENCE LISTING

&lt;110&gt; Garcia-Calvo, Margarita

&lt;120&gt; NPC1L1 (NPC3) AND METHODS OF IDENTIFYING LIGANDS THEREOF

&lt;130&gt; 36134-PCT 074669.0111

&lt;140&gt; To Be Assigned

&lt;141&gt; 2005-01-14

&lt;150&gt; US 60/537,341

&lt;151&gt; 2004-01-16

&lt;160&gt; 51

&lt;170&gt; PatentIn version 3.1

&lt;210&gt; 1

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agc	ctc	tcc	gac	tgc	tgt	gtc	aac	agc	ctc	ctt	cag	tac	ttc	cag	aac	1488	
Ser	Leu	Ser	Asp	Cys	Cys	Val	Asn	Ser	Leu	Leu	Gln	Tyr	Phe	Gln	Asn		
				485					490					495			
aac	cgc	acc	ctc	ctg	atg	ctc	acg	gcc	aac	cag	act	ctg	aac	ggc	cag	1536	
Asn	Arg	Thr	Leu	Leu	Met	Leu	Thr	Ala	Asn	Gln	Thr	Leu	Asn	Gly	Gln		
			500					505						510			

acc tcc ctg gtg gac tgg aag gac cat ttc ctc tac tgt gca aat gcc Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala 515 520 525	1584
cct ctc acg ttc aaa gat ggc acg tct ctg gcc ctg agc tgc atg gct Pro Leu Thr Phe Lys Asp Gly Thr Ser Leu Ala Leu Ser Cys Met Ala 530 535 540	1632
gac tac ggg gct cct gtc ttc ccc ttc ctt gct gtt ggg gga tac caa Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln 545 550 555 560	1680
ggc acg gac tat tcc gag gca gaa gcg ctg atc ata acc ttc tct ctc Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Leu 565 570 575	1728
aat aac tac ccc gct gat gat ccc cgc atg gcc cag gcc aag ctc tgg Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala Gln Ala Lys Leu Trp 580 585 590	1776
gag gag gct ttc ttg aag gaa atg gaa tcc ttc cag agg aac aca agt Glu Glu Ala Phe Leu Lys Glu Met Glu Ser Phe Gln Arg Asn Thr Ser 595 600 605	1824
gac aag ttc cag gtt gcg ttc tca gct gag cgc tct ctg gag gat gag Asp Lys Phe Gln Val Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu 610 615 620	1872
atc aac cgc acc acc atc cag gac ctg cct gtc ttt gcc gtc agc tac Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Val Ser Tyr 625 630 635 640	1920
att atc gtc ttc ctg tac atc tcc ctg gcc ctg ggc agc tac tcc aga Ile Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg 645 650 655	1968
tgc agc cga gta gcg gtg gag tcc aag gct act ctg ggc cta ggt ggg Cys Ser Arg Val Ala Val Glu Ser Lys Ala Thr Leu Gly Leu Gly Gly 660 665 670	2016
gtg att gtt gtg ctg gga gca gtt ctg gct gcc atg ggc ttc tac tcc Val Ile Val Val Leu Gly Ala Val Leu Ala Ala Met Gly Phe Tyr Ser 675 680 685	2064
tac ctg ggt gtc ccc tct tct ctg gtt atc atc caa gtg gta cct ttc Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe 690 695 700	2112
ctg gtg cta gct gtg gga gct gac aac atc ttc atc ttt gtt ctt gag Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu 705 710 715 720	2160
tac cag agg cta cct agg atg cct ggg gaa cag cga gag gct cac att Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile 725 730 735	2208
ggc cgc acc ctg ggc agt gtg gcc ccc agc atg ctg ctg tgc agc ctc Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu 740 745 750	2256

tct gag gcc atc tgc ttc ttt cta ggg gcc ctg acc ccc atg cca gct Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala 755 760 765	2304
gtg agg acc ttc gcc ttg acc tct ggc tta gca att atc ctc gac ttc Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Leu Asp Phe 770 775 780	2352
ctg ctc cag atg act gcc ttt gtg gcc ctg ctc tcc ctg gat agc aag Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys 785 790 795 800	2400
agg cag gag gcc tct cgc ccg gat gtc tta tgc tgc ttt tca acc cgg Arg Gln Glu Ala Ser Arg Pro Asp Val Leu Cys Cys Phe Ser Thr Arg 805 810 815	2448
aag ctg ccc cca cct aaa gaa aaa gaa ggc ctc tta ctc cgc ttc ttc Lys Leu Pro Pro Lys Glu Lys Glu Gly Leu Leu Leu Arg Phe Phe 820 825 830	2496
cgc aag ata tac gct cct ttc ctg ctg cac aga ttc atc cgc cct gtt Arg Lys Ile Tyr Ala Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val 835 840 845	2544
gtg atg ctg ctg ttt ctg acc ctg ttt gga gca aat ctc tac tta atg Val Met Leu Leu Phe Leu Thr Leu Phe Gly Ala Asn Leu Tyr Leu Met 850 855 860	2592
tgc aac atc aac gtg ggg cta gac cag gag ctg gct ctg ccc aag gac Cys Asn Ile Asn Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp 865 870 875 880	2640
tcg tac ttg ata gac tac ttc ctc ttt ctg aac cga tac ctt gaa gtg Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val 885 890 895	2688
ggg cct cca gtg tac ttt gtc acc acc tcg ggc ttc aac ttc tcc agc Gly Pro Pro Val Tyr Phe Val Thr Thr Ser Gly Phe Asn Phe Ser Ser 900 905 910	2736
gag gca ggc atg aac gcc act tgc tct agc gca ggc tgt aag agc ttc Glu Ala Gly Met Asn Ala Thr Cys Ser Ser Ala Gly Cys Lys Ser Phe 915 920 925	2784
tcc cta acc cag aaa atc cag tat gcc agt gaa ttc cct gac cag tct Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asp Gln Ser 930 935 940	2832
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ctg acc ccg tcc tcc tcc tgc tgt cgc ctt tat ata cgt ggc ccc cat Leu Thr Pro Ser Ser Ser Cys Cys Arg Leu Tyr Ile Arg Gly Pro His 965 970 975	2928
aag gat gag ttc tgt ccc tca acg gat act tcc ttc aac tgc tta aaa Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys	2976

980	985	990	
aac tgc atg aac cgc act ctg ggt cct gtg agg ccc aca gcg gaa cag Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr Ala Glu Gln 995 1000 1005			3024
ttt cat aag tac ctg ccc tgg ttc ctg aat gat ccg ccc aat atc Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Pro Pro Asn Ile 1010 1015 1020			3069
aga tgt ccc aaa ggg ggt cta gca gcg tat aga acg tct gtg aat Arg Cys Pro Lys Gly Gly Leu Ala Ala Tyr Arg Thr Ser Val Asn 1025 1030 1035			3114
ttg agc tca gat ggc cag gtt ata gcc tcc cag ttc atg gcc tac Leu Ser Ser Asp Gly Gln Val Ile Ala Ser Gln Phe Met Ala Tyr 1040 1045 1050			3159
cac aag ccc tta agg aac tca cag gac ttc aca gaa gct ctc cgg His Lys Pro Leu Arg Asn Ser Gln Asp Phe Thr Glu Ala Leu Arg 1055 1060 1065			3204
gcg tcc cgg ttg cta gca gcc aac atc aca gct gac cta cgg aag Ala Ser Arg Leu Leu Ala Ala Asn Ile Thr Ala Asp Leu Arg Lys 1070 1075 1080			3249
gtg cct ggg aca gat cca aac ttt gag gtc ttc cct tac acg atc Val Pro Gly Thr Asp Pro Asn Phe Glu Val Phe Pro Tyr Thr Ile 1085 1090 1095			3294
tcc aac gtg ttc tac cag caa tac ctg acg gtc ctt cct gag gga Ser Asn Val Phe Tyr Gln Gln Tyr Leu Thr Val Leu Pro Glu Gly 1100 1105 1110			3339
atc ttc acc ctt gct ctt tgc ttt gtg ccc acc ttt gtt gtc tgc Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys 1115 1120 1125			3384
tac ctc cta ctg ggc ctg gac atg tgc tca ggg atc ctc aac cta Tyr Leu Leu Leu Gly Leu Asp Met Cys Ser Gly Ile Leu Asn Leu 1130 1135 1140			3429
ctc tcc atc att atg att ctc gtg gac acc att ggc ctc atg gct Leu Ser Ile Ile Met Ile Leu Val Asp Thr Ile Gly Leu Met Ala 1145 1150 1155			3474
gtg tgg ggt atc agc tat aat gcg gta tcc ctc atc aac ctt gtc Val Trp Gly Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val 1160 1165 1170			3519
acg gca gtg ggc atg tct gtg gag ttt gtg tcc cac atc act cgg Thr Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg 1175 1180 1185			3564
tcc ttt gct gta agc acc aag cct acc cgg ctg gag agg gct aaa Ser Phe Ala Val Ser Thr Lys Pro Thr Arg Leu Glu Arg Ala Lys 1190 1195 1200			3609
gat gct act gtc ttc atg ggc agt gcg gtg ttt gct gga gtg gcc			3654

Asp Ala Thr Val Phe Met Gly Ser Ala Val Phe Ala Gly Val Ala  
 1205 1210 1215  
 atg acc aac ttc cca ggc atc ctc atc ttg ggc ttt gcc caa gcc 3699  
 Met Thr Asn Phe Pro Gly Ile Leu Ile Leu Gly Phe Ala Gln Ala  
 1220 1225 1230  
 cag ctt att cag atc ttc ttc ttc cgc ctc aac ctt ctg atc acc 3744  
 Gln Leu Ile Gln Ile Phe Phe Phe Arg Leu Asn Leu Ile Thr  
 1235 1240 1245  
 ttg ctg ggt ctg ctg cat ggc ctg gtc ttc ctg ccg gtt gtc ctc 3789  
 Leu Leu Gly Leu Leu His Gly Leu Val Phe Leu Pro Val Val Leu  
 1250 1255 1260  
 agc tat ctg gga cca gat gtt aac caa gct ctg gta cag gag gag 3834  
 Ser Tyr Leu Gly Pro Asp Val Asn Gln Ala Leu Val Gln Glu Glu  
 1265 1270 1275  
 aaa cta gcc agc gag gca gca gtg gcc cca gag cct tct tgc cca 3879  
 Lys Leu Ala Ser Glu Ala Ala Val Ala Pro Glu Pro Ser Cys Pro  
 1280 1285 1290  
 cag tac ccc tcc cct gct gat gcg gat gcc aat gtt aac tac gcc 3924  
 Gln Tyr Pro Ser Pro Ala Asp Ala Asp Ala Asn Val Asn Tyr Gly  
 1295 1300 1305  
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 Phe Ala Pro Glu Leu Ala His Gly Ala Asn Ala Ala Arg Ser Ser  
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&lt;211&gt; 1331

&lt;212&gt; PRT

&lt;213&gt; Rattus sp.

&lt;400&gt; 2

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 Ala Ala Gln Gly Glu Leu Tyr Thr Pro Lys His Glu Ala Gly Val Cys  
 20 25 30  
 Thr Phe Tyr Glu Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Gly Leu  
 35 40 45



Thr Ser Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg His  
50 55 60

Val Thr Gly Glu His Leu Ala Leu Leu Gln Arg Ile Cys Pro Arg Leu  
65 70 75 80

Tyr Asn Gly Pro Asn Thr Thr Phe Ala Cys Cys Ser Thr Lys Gln Leu  
85 90 95

Leu Ser Leu Glu Ser Ser Met Ser Ile Thr Lys Ala Leu Leu Thr Arg  
100 105 110

Cys Pro Ala Cys Ser Asp Asn Phe Val Ser Leu His Cys His Asn Thr  
115 120 125

Cys Ser Pro Asp Gln Ser Leu Phe Ile Asn Val Thr Arg Val Val Glu  
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Arg Gly Ala Gly Glu Pro Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr  
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Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Gln Val Arg  
165 170 175

Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr  
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Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp  
195 200 205

Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu  
210 215 220

Pro Gly Gln Ala Leu Pro Asp Gly Ile Gln Pro Leu Asn Gly Lys Ile  
225 230 235 240

Ala Pro Cys Asn Glu Ser Gln Gly Asp Asp Ser Ala Val Cys Ser Cys  
245 250 255

Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Glu Ala Leu  
260 265 270

Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile  
275 280 285

Ile Ile Phe Thr Ala Val Phe Val Leu Leu Ser Ala Val Leu Val Arg  
 290 295 300  
 Leu Arg Val Val Ser Asn Arg Asn Lys Asn Lys Ala Glu Gly Pro Gln  
 305 310 315 320  
 Glu Ala Pro Lys Leu Pro His Lys His Lys Leu Ser Pro His Thr Ile  
 325 330 335  
 Leu Gly Arg Phe Phe Gln Asn Trp Gly Thr Arg Val Ala Ser Trp Pro  
 340 345 350  
 Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ala Ala  
 355 360 365  
 Gly Leu Thr Phe Ile Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser  
 370 375 380  
 Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ser Phe His Asp Glu His  
 385 390 395 400  
 Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Arg Asn  
 405 410 415  
 Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Ser Lys Asn Phe  
 420 425 430  
 Ser Gly Ile Leu Ser Leu Asp Phe Leu Leu Glu Leu Leu Glu Leu Gln  
 435 440 445  
 Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Glu Arg Asn  
 450 455 460  
 Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Tyr Asn Thr  
 465 470 475 480  
 Ser Leu Ser Asp Cys Cys Val Asn Ser Leu Leu Gln Tyr Phe Gln Asn  
 485 490 495  
 Asn Arg Thr Leu Leu Met Leu Thr Ala Asn Gln Thr Leu Asn Gly Gln  
 500 505 510  
 Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala

515	520	525
Pro Leu Thr Phe Lys Asp Gly Thr Ser Leu Ala Leu Ser Cys Met Ala		
530	535	540
Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln		
545	550	555
Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Leu		
565	570	575
Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala Gln Ala Lys Leu Trp		
580	585	590
Glu Glu Ala Phe Leu Lys Glu Met Glu Ser Phe Gln Arg Asn Thr Ser		
595	600	605
Asp Lys Phe Gln Val Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu		
610	615	620
Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Val Ser Tyr		
625	630	635
Ile Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg		
645	650	655
Cys Ser Arg Val Ala Val Glu Ser Lys Ala Thr Leu Gly Leu Gly Gly		
660	665	670
Val Ile Val Val Leu Gly Ala Val Leu Ala Ala Met Gly Phe Tyr Ser		
675	680	685
Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe		
690	695	700
Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu		
705	710	715
Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile		
725	730	735
Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu		
740	745	750

Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala  
 755 760 765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Leu Asp Phe  
 770 775 780

Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys  
 785 790 795 800

Arg Gln Glu Ala Ser Arg Pro Asp Val Leu Cys Cys Phe Ser Thr Arg  
 805 810 815

Lys Leu Pro Pro Pro Lys Glu Lys Glu Gly Leu Leu Leu Arg Phe Phe  
 820 825 830

Arg Lys Ile Tyr Ala Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val  
 835 840 845

Val Met Leu Leu Phe Leu Thr Leu Phe Gly Ala Asn Leu Tyr Leu Met  
 850 855 860

Cys Asn Ile Asn Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp  
 865 870 875 880

Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val  
 885 890 895

Gly Pro Pro Val Tyr Phe Val Thr Thr Ser Gly Phe Asn Phe Ser Ser  
 900 905 910

Glu Ala Gly Met Asn Ala Thr Cys Ser Ser Ala Gly Cys Lys Ser Phe  
 915 920 925

Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asp Gln Ser  
 930 935 940

Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp  
 945 950 955 960

Leu Thr Pro Ser Ser Ser Cys Cys Arg Leu Tyr Ile Arg Gly Pro His  
 965 970 975

Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys  
 980 985 990

Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr Ala Glu Gln  
995 1000 1005

Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Pro Pro Asn Ile  
1010 1015 1020

Arg Cys Pro Lys Gly Gly Leu Ala Ala Tyr Arg Thr Ser Val Asn  
1025 1030 1035

Leu Ser Ser Asp Gly Gln Val Ile Ala Ser Gln Phe Met Ala Tyr  
1040 1045 1050

His Lys Pro Leu Arg Asn Ser Gln Asp Phe Thr Glu Ala Leu Arg  
1055 1060 1065

Ala Ser Arg Leu Leu Ala Ala Asn Ile Thr Ala Asp Leu Arg Lys  
1070 1075 1080

Val Pro Gly Thr Asp Pro Asn Phe Glu Val Phe Pro Tyr Thr Ile  
1085 1090 1095

Ser Asn Val Phe Tyr Gln Gln Tyr Leu Thr Val Leu Pro Glu Gly  
1100 1105 1110

Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys  
1115 1120 1125

Tyr Leu Leu Leu Gly Leu Asp Met Cys Ser Gly Ile Leu Asn Leu  
1130 1135 1140

Leu Ser Ile Ile Met Ile Leu Val Asp Thr Ile Gly Leu Met Ala  
1145 1150 1155

Val Trp Gly Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val  
1160 1165 1170

Thr Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg  
1175 1180 1185

Ser Phe Ala Val Ser Thr Lys Pro Thr Arg Leu Glu Arg Ala Lys  
1190 1195 1200

Asp Ala Thr Val Phe Met Gly Ser Ala Val Phe Ala Gly Val Ala  
1205 1210 1215

Met Thr Asn Phe Pro Gly Ile Leu Ile Leu Gly Phe Ala Gln Ala  
1220 1225 1230

Gln Leu Ile Gln Ile Phe Phe Phe Arg Leu Asn Leu Leu Ile Thr  
1235 1240 1245

Leu Leu Gly Leu Leu His Gly Leu Val Phe Leu Pro Val Val Leu  
1250 1255 1260

Ser Tyr Leu Gly Pro Asp Val Asn Gln Ala Leu Val Gln Glu Glu  
1265 1270 1275

Lys Leu Ala Ser Glu Ala Ala Val Ala Pro Glu Pro Ser Cys Pro  
1280 1285 1290

Gln Tyr Pro Ser Pro Ala Asp Ala Asp Ala Asn Val Asn Tyr Gly  
1295 1300 1305

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Leu Pro Lys Ser Asp Gln Lys Phe  
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Met Ala Glu Ala Gly Leu Arg Gly Trp Leu Leu Trp Ala Leu Leu Leu  
1 5 10 15

48

cgc ttg gcc cag agt gag cct tac aca acc atc cac cag cct ggc tac Arg Leu Ala Gln Ser Glu Pro Tyr Thr Thr Ile His Gln Pro Gly Tyr 20 25 30	96
tgc gcc ttc tat gac gaa tgt ggg aag aac cca gag ctg tct gga agc Cys Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser 35 40 45	144
ctc atg aca ctc tcc aac gtg tcc tgc ctg tcc aac acg ccg gcc cgc Leu Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg 50 55 60	192
aag atc aca ggt gat cac ctg atc cta tta cag aag atc tgc ccc cgc Lys Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg 65 70 75 80	240
ctc tac acc ggc ccc aac acc caa gcc tgc tgc tcc gcc aag cag ctg Leu Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu 85 90 95	288
gta tca ctg gaa gcg agt ctg tgc atc acc aag gcc ctc ctc acc cgc Val Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg 100 105 110	336
tgc cca gcc tgc tct gac aat ttt gtg aac ctg cac tgc cac aac acg Cys Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr 115 120 125	384
tgc agc ccc aat cag agc ctc ttc atc aat gtg acc cgc gtg gcc cag Cys Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln 130 135 140	432
cta ggg gct gga caa ctc cca gct gtg gtg gcc tat gag gcc ttc tac Leu Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr 145 150 155 160	480
cag cat agc ttt gcc gag cag agc tat gac tcc tgc agc cgt gtg cgc Gln His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg 165 170 175	528
gtc cct gca gct gcc acg ctg gct gtg ggc acc atg tgt ggc gtg tat Val Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr 180 185 190	576
ggc tct gcc ctt tgc aat gcc cag cgc tgg ctc aac ttc cag gga gac Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp 195 200 205	624
aca ggc aat ggt ctg gcc cca ctg gac atc acc ttc cac ctc ttg gag Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu 210 215 220	672
cct ggc cag gcc gtg ggg agt ggg att cag cct ctg aat gag ggg gtt Pro Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val 225 230 235 240	720
gca cgt tgc aat gag tcc caa ggt gac gac gtg gcg acc tgc tcc tgc Ala Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys 245 250 255	768

caa gac tgt gct gca tcc tgt cct gcc ata gcc cgc ccc cag gcc ctc Gln Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu 260 265 270	816
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ttc cgt gtg gcc ccc gcc agg gac aaa agc aag atg gtg gac ccc aag Phe Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys 305 310 315 320	960
aag ggc acc agc ctc tct gac aag ctc agc ttc tcc acc cac acc ctc Lys Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu 325 330 335	1008
ctt ggc cag ttc ttc cag ggc tgg ggc acg tgg gtg gct tcg tgg cct Leu Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro 340 345 350	1056
ctg acc atc ttg gtg cta tct gtc atc ccg gtg gtg gcc ttg gca gcg Leu Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala 355 360 365	1104
ggc ctg gtc ttt aca gaa ctc act acg gac ccc gtg gag ctg tgg tcg Gly Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser 370 375 380	1152
gcc ccc aac agc caa gcc cgg agt gag aaa gct ttc cat gac cag cat Ala Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His 385 390 395 400	1200
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cgg tcc agc tac agg tat gac tct ctg ctg ctg ggg ccc aag aac ttc Arg Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe 420 425 430	1296
agc gga atc ctg gac ctg gac ttg ctg ctg gag ctg cta gag ctg cag Ser Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Glu Leu Gln 435 440 445	1344
gag agg ctg cgg cac ctc cag gta tgg tcg ccc gaa gca cag cgc aac Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn 450 455 460	1392
atc tcc ctg cag gac atc tgc tac gcc ccc ctc aat ccg gac aat acc Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr 465 470 475 480	1440
agt ctc tac gac tgc tgc atc aac agc ctc ctg cag tat ttc cag aac Ser Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn	1488



485	490	495	
aac cgc acg ctc ctg ctg ctc aca gcc aac cag aca ctg atg ggg cag Asn Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln 500 505 510			1536
acc tcc caa gtc gac tgg aag gac cat ttt ctg tac tgt gcc aat gcc Thr Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala 515 520 525			1584
ccg ctc acc ttc aag gat ggc aca gcc ctg gcc ctg agc tgc atg gct Pro Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala 530 535 540			1632
gac tac ggg gcc cct gtc ttc ccc ttc ctt gcc att ggg ggg tac aaa Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys 545 550 555 560			1680
gga aag gac tat tct gag gca gag gcc ctg atc atg acg ttc tcc ctc Gly Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu 565 570 575			1728
aac aat tac cct gcc ggg gac ccc cgt ctg gcc cag gcc aag ctg tgg Asn Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp 580 585 590			1776
gag gag gcc ttc tta gag gaa atg cga gcc ttc cag cgt cgg atg gct Glu Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala 595 600 605			1824
ggc atg ttc cag gtc acg ttc acg gct gag cgc tct ctg gaa gac gag Gly Met Phe Gln Val Thr Phe Thr Ala Glu Arg Ser Leu Glu Asp Glu 610 615 620			1872
atc aat cgc acc aca gct gaa gac ctg ccc atc ttt gcc acc agc tac Ile Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr 625 630 635 640			1920
att gtc ata ttc ctg tac atc tct ctg gcc ctg ggc agc tat tcc agc Ile Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser 645 650 655			1968
tgg agc cga gtg atg gtg gac tcc aag gcc acg ctg ggc ctc ggc ggg Trp Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly 660 665 670			2016
gtg gcc gtg gtc ctg gga gca gtc atg gct gcc atg ggc ttc ttc tcc Val Ala Val Val Leu Gly Ala Val Met Ala Ala Met Gly Phe Phe Ser 675 680 685			2064
tac ttg ggt atc cgc tcc tcc ctg gtc atc ctg caa gtg gtt cct ttc Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe 690 695 700			2112
ctg gtg ctg tcc gtg ggg gct gat aac atc ttc atc ttt gtt ctc gag Leu Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu 705 710 715 720			2160
tac cag agg ctg ccc cgg agg cct ggg gag cca cga gag gtc cac att			2208

Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile	
725 730 735	
ggg cga gcc cta ggc agg gtg gct ccc agc atg ctg ttg tgc agc ctc	2256
Gly Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu	
740 745 750	
tct gag gcc atc tgc ttc ttc cta ggg gcc ctg acc ccc atg cca gct	2304
Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala	
755 760 765	
gtg cgg acc ttt gcc ctg acc tct ggc ctt gca gtg atc ctt gac ttc	2352
Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe	
770 775 780	
ctc ctg cag atg tca gcc ttt gtg gcc ctg ctc tcc ctg gac agc aag	2400
Leu Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys	
785 790 795 800	
agg cag gag gcc tcc cgg ttg gac gtc tgc tgc tgt gtc aag ccc cag	2448
Arg Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln	
805 810 815	
gag ctg ccc ccg cct ggc cag gga gag ggg ctc ctg ctt ggc ttc ttc	2496
Glu Leu Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe	
820 825 830	
caa aag gct tat gcc ccc ttc ctg ctg cac tgg atc act cga ggt gtt	2544
Gln Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val	
835 840 845	
gtg ctg ctg ctg ttt ctc gcc ctg ttc gga gtg agc ctc tac tcc atg	2592
Val Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met	
850 855 860	
tgc cac atc agc gtg gga ctg gac cag gag ctg gcc ctg ccc aag gac	2640
Cys His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp	
865 870 875 880	
tcg tac ctg ctt gac tat ttc ctc ttt ctg aac cgc tac ttc gag gtg	2688
Ser Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val	
885 890 895	
ggg gcc ccg gtg tac ttt gtt acc acc ttg ggc tac aac ttc tcc agc	2736
Gly Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser	
900 905 910	
gag gct ggg atg aat gcc atc tgc tcc agt gca ggc tgc aac aac ttc	2784
Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe	
915 920 925	
tcc ttc acc cag aag atc cag tat gcc aca gag ttc cct gag cag tct	2832
Ser Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser	
930 935 940	
tac ctg gcc atc cct gcc tcc tcc tgg gtg gat gac ttc att gac tgg	2880
Tyr Leu Ala Ile Pro Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp	
945 950 955 960	

ctg acc ccg tcc tcc tgc tgc cgc ctt tat ata tct ggc ccc aat aag Leu Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys 965 970 975	2928
gac aag ttc tgc ccc tgc acc gtc aac tct ctg aac tgc cta aag aac Asp Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn 980 985 990	2976
tgc atg agc atc acg atg ggc tct gtg agg ccc tgc gtg gag cag ttc Cys Met Ser Ile Thr Met Gly Ser Val Arg Pro Ser Val Glu Gln Phe 995 1000 1005	3024
cat aag tat ctt ccc tgg ttc ctg aac gac cgg ccc aac atc aaa His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Arg Pro Asn Ile Lys 1010 1015 1020	3069
tgt ccc aaa ggc ggc ctg gca gca tac agc acc tct gtg aac ttg Cys Pro Lys Gly Gly Leu Ala Ala Tyr Ser Thr Ser Val Asn Leu 1025 1030 1035	3114
act tca gat ggc cag gtt tta gcc tcc agg ttc atg gcc tat cac Thr Ser Asp Gly Gln Val Leu Ala Ser Arg Phe Met Ala Tyr His 1040 1045 1050	3159
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gct cga gag ctg gca gcc aac atc act gct gac ctg cgg aaa gtg Ala Arg Glu Leu Ala Ala Asn Ile Thr Ala Asp Leu Arg Lys Val 1070 1075 1080	3249
cct gga aca gac ccg gct ttt gag gtc ttc ccc tac acg atc acc Pro Gly Thr Asp Pro Ala Phe Glu Val Phe Pro Tyr Thr Ile Thr 1085 1090 1095	3294
aat gtg ttt tat gag cag tac ctg acc atc ctc cct gag ggg ctc Asn Val Phe Tyr Glu Gln Tyr Leu Thr Ile Leu Pro Glu Gly Leu 1100 1105 1110	3339
ttc atg ctc agc ctc tgc ctt gtg ccc acc ttc gct gtc tcc tgc Phe Met Leu Ser Leu Cys Leu Val Pro Thr Phe Ala Val Ser Cys 1115 1120 1125	3384
ctc ctg ctg ggc ctg gac ctg cgc tcc ggc ctc ctc aac ctg ctc Leu Leu Leu Gly Leu Asp Leu Arg Ser Gly Leu Leu Asn Leu Leu 1130 1135 1140	3429
tcc att gtc atg atc ctc gtg gac act gtc ggc ttc atg gcc ctg Ser Ile Val Met Ile Leu Val Asp Thr Val Gly Phe Met Ala Leu 1145 1150 1155	3474
tgg gac atc agt tac aat gct gtg tcc ctc atc aac ctg gtc tgc Trp Asp Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val Ser 1160 1165 1170	3519
gcg gtg ggc atg tct gtg gag ttt gtg tcc cac att acc cgc tcc Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg Ser 1175 1180 1185	3564

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 Phe Ala Ile Ser Thr Lys Pro Thr Trp Leu Glu Arg Ala Lys Glu  
 1190 1195 1200  
 gcc acc atc tct atg gga agt gcg gtg ttt gca ggt gtg gcc atg 3654  
 Ala Thr Ile Ser Met Gly Ser Ala Val Phe Ala Gly Val Ala Met  
 1205 1210 1215  
 acc aac ctg cct ggc atc ctt gtc ctg ggc ctc gcc aag gcc cag 3699  
 Thr Asn Leu Pro Gly Ile Leu Val Leu Gly Leu Ala Lys Ala Gln  
 1220 1225 1230  
 ctc att cag atc ttc ttc ttc cgc ctc aac ctc ctg atc act ctg 3744  
 Leu Ile Gln Ile Phe Phe Phe Arg Leu Asn Leu Leu Ile Thr Leu  
 1235 1240 1245  
 ctg ggc ctg ctg cat ggc ttg gtc ttc ctg ccc gtc atc ctc agc 3789  
 Leu Gly Leu Leu His Gly Leu Val Phe Leu Pro Val Ile Leu Ser  
 1250 1255 1260  
 tac gtg ggg cct gac gtt aac ccg gct ctg gca ctg gag cag aag 3834  
 Tyr Val Gly Pro Asp Val Asn Pro Ala Leu Ala Leu Glu Gln Lys  
 1265 1270 1275  
 cgg gct gag gag gcg gtg gca gca gtc atg gtg gcc tct tgc cca 3879  
 Arg Ala Glu Glu Ala Val Ala Ala Val Met Val Ala Ser Cys Pro  
 1280 1285 1290  
 aat cac ccc tcc cga gtc tcc aca gct gac aac atc tat gtc aac 3924  
 Asn His Pro Ser Arg Val Ser Thr Ala Asp Asn Ile Tyr Val Asn  
 1295 1300 1305  
 cac agc ttt gaa ggt tct atc aaa ggt gct ggt gcc atc agc aac 3969  
 His Ser Phe Glu Gly Ser Ile Lys Gly Ala Gly Ala Ile Ser Asn  
 1310 1315 1320  
 ttc ttg ccc aac aat ggg cgg cag ttc tga 3999  
 Phe Leu Pro Asn Asn Gly Arg Gln Phe  
 1325 1330

&lt;210&gt; 4

&lt;211&gt; 1332

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 4

Met Ala Glu Ala Gly Leu Arg Gly Trp Leu Leu Trp Ala Leu Leu Leu  
 1 5 10 15

Arg Leu Ala Gln Ser Glu Pro Tyr Thr Thr Ile His Gln Pro Gly Tyr

20	25	30
Cys Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser		
35	40	45
Leu Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg		
50	55	60
Lys Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg		
65	70	75
Leu Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu		
85	90	95
Val Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg		
100	105	110
Cys Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr		
115	120	125
Cys Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln		
130	135	140
Leu Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr		
145	150	155
Gln His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg		
165	170	175
Val Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr		
180	185	190
Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp		
195	200	205
Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu		
210	215	220
Pro Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val		
225	230	235
Ala Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys		
245	250	255

Gln Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu  
260 265 270

Asp Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile  
275 280 285

Ile Ile Leu Cys Ser Val Phe Ala Val Val Thr Ile Leu Leu Val Gly  
290 295 300

Phe Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys  
305 310 315 320

Lys Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu  
325 330 335

Leu Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro  
340 345 350

Leu Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala  
355 360 365

Gly Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser  
370 375 380

Ala Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His  
385 390 395 400

Phe Gly Pro Phe Phe Arg Thr Asn Gln Val Ile Leu Thr Ala Pro Asn  
405 410 415

Arg Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe  
420 425 430

Ser Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln  
435 440 445

Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn  
450 455 460

Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr  
465 470 475 480

Ser Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn  
485 490 495

Asn Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln  
 500 505 510

Thr Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala  
 515 520 525

Pro Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala  
 530 535 540

Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys  
 545 550 555 560

Gly Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu  
 565 570 575

Asn Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp  
 580 585 590

Glu Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala  
 595 600 605

Gly Met Phe Gln Val Thr Phe Thr Ala Glu Arg Ser Leu Glu Asp Glu  
 610 615 620

Ile Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr  
 625 630 635 640

Ile Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser  
 645 650 655

Trp Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly  
 660 665 670

Val Ala Val Val Leu Gly Ala Val Met Ala Ala Met Gly Phe Phe Ser  
 675 680 685

Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe  
 690 695 700

Leu Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu  
 705 710 715 720

Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile  
 725 730 735

Gly Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu  
                   740                                  745                                  750

Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala  
                   755                                  760                                  765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe  
                   770                                  775                                  780

Leu Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys  
                   785                                  790                                  795                                  800

Arg Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln  
                                   805                                  810                                  815

Glu Leu Pro Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe  
                                   820                                  825                                  830

Gln Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val  
                   835                                  840                                  845

Val Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met  
                   850                                  855                                  860

Cys His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp  
                   865                                  870                                  875                                  880

Ser Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val  
                                   885                                  890                                  895

Gly Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser  
                   900                                  905                                  910

Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe  
                   915                                  920                                  925

Ser Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser  
                   930                                  935                                  940

Tyr Leu Ala Ile Pro Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp  
                   945                                  950                                  955                                  960

Leu Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys



965	970	975
Asp Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn		
980	985	990
Cys Met Ser Ile Thr Met Gly Ser Val Arg Pro Ser Val Glu Gln Phe		
995	1000	1005
His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Arg Pro Asn Ile Lys		
1010	1015	1020
Cys Pro Lys Gly Gly Leu Ala Ala Tyr Ser Thr Ser Val Asn Leu		
1025	1030	1035
Thr Ser Asp Gly Gln Val Leu Ala Ser Arg Phe Met Ala Tyr His		
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Lys Pro Leu Lys Asn Ser Gln Asp Tyr Thr Glu Ala Leu Arg Ala		
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Ala Arg Glu Leu Ala Ala Asn Ile Thr Ala Asp Leu Arg Lys Val		
1070	1075	1080
Pro Gly Thr Asp Pro Ala Phe Glu Val Phe Pro Tyr Thr Ile Thr		
1085	1090	1095
Asn Val Phe Tyr Glu Gln Tyr Leu Thr Ile Leu Pro Glu Gly Leu		
1100	1105	1110
Phe Met Leu Ser Leu Cys Leu Val Pro Thr Phe Ala Val Ser Cys		
1115	1120	1125
Leu Leu Leu Gly Leu Asp Leu Arg Ser Gly Leu Leu Asn Leu Leu		
1130	1135	1140
Ser Ile Val Met Ile Leu Val Asp Thr Val Gly Phe Met Ala Leu		
1145	1150	1155
Trp Asp Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val Ser		
1160	1165	1170
Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg Ser		
1175	1180	1185

Phe Ala Ile Ser Thr Lys Pro Thr Trp Leu Glu Arg Ala Lys Glu  
 1190 1195 1200  
 Ala Thr Ile Ser Met Gly Ser Ala Val Phe Ala Gly Val Ala Met  
 1205 1210 1215  
 Thr Asn Leu Pro Gly Ile Leu Val Leu Gly Leu Ala Lys Ala Gln  
 1220 1225 1230  
 Leu Ile Gln Ile Phe Phe Phe Arg Leu Asn Leu Leu Ile Thr Leu  
 1235 1240 1245  
 Leu Gly Leu Leu His Gly Leu Val Phe Leu Pro Val Ile Leu Ser  
 1250 1255 1260  
 Tyr Val Gly Pro Asp Val Asn Pro Ala Leu Ala Leu Glu Gln Lys  
 1265 1270 1275  
 Arg Ala Glu Glu Ala Val Ala Ala Val Met Val Ala Ser Cys Pro  
 1280 1285 1290  
 Asn His Pro Ser Arg Val Ser Thr Ala Asp Asn Ile Tyr Val Asn  
 1295 1300 1305  
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 1325 1330

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<211> 885

<212> DNA

<213> Rattus sp.

<400> 5

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 gcctccttca gtacttccag aacaaccgca cctcctgat gctcacggcc aaccagactc 180  
 tgaatggcca gacctcctg gtggactgga aggaccattt cctctactgt gcaaatgccc 240

ctctcacgtt caaagatggc acgtctctgg ccctgagctg catggctgac tacggggctc 300  
ctgtcttccc cttccttgct gttgggggat accaaggcac ggactattcc gaggcagaag 360  
cgctgatcat aaccttctct ctcaataact accccgctga tgatccccgc atggcccagg 420  
ccaagctctg ggaggaggct ttcttgaagg aaatggaatc cttccagagg aacacaagtg 480  
acaagttcca ggttgcgttc tcagctgagc gctctctgga ggatgagatc aaccgcacca 540  
ccatccagga cctgcctgtc tttgccgtca gctacattat cgtcttctg tacatctccc 600  
tgccctggg cagctactcc agatgcagcc gagtagcggg ggagtccaag gctactctgg 660  
gcctaggtgg ggtgatagtg tgctgggagc agttctggct tgcattgggc ttctaactcc 720  
tacctgggtg tcccctcttc tctggttatc atccaagtgg tacctttcct ggtgcttaag 780  
ctgtgggagc tggacacatc tacatcctag acttgagtac cagaggtagc taggaagccg 840  
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<210> 6

<211> 458

<212> DNA

<213> Rattus sp.

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<210> 7

<211> 896

<212> DNA

<213> Rattus sp.

&lt;400&gt; 7

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aggcatcctc atcttggggg ctttgcccca agccagggct tattcagatc ttcttcttcc	720
gcctcaacct tctgatcacc tttgctggg tctgctgcat ggctggtctt cctgcccggg	780
ttgtcctcag ctatctggga ccagatgtaa ccaaggctct gctaccgga ggagaaacta	840
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&lt;210&gt; 8

&lt;211&gt; 3124

&lt;212&gt; DNA

&lt;213&gt; Rattus sp.

&lt;400&gt; 8

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ccctggtgga ctggaaggac catttctct actgtgcaaa tgcccctctc acgttcaaag	240
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gaa gcc ccc aac ctc cct cgt aag cgc aga ttc tca cct cac act gtc Glu Ala Pro Asn Leu Pro Arg Lys Arg Arg Phe Ser Pro His Thr Val 325 330 335	1008
ctt ggc cgg ttc ttc gag agc tgg gga aca agg gtg gcc tca tgg cca Leu Gly Arg Phe Phe Glu Ser Trp Gly Thr Arg Val Ala Ser Trp Pro 340 345 350	1056
ctc act gtc ttg gca ctg tcc ttc ata gtt gtg ata gcc ttg tca gta Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ser Val 355 360 365	1104
ggc ctg acc ttt ata gaa ctc acc aca gac cct gtg gaa ctg tgg tcg Gly Leu Thr Phe Ile Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser 370 375 380	1152
gcc cct aaa agc caa gcc cgg aaa gaa aag gct ttc cat gac gag cat Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ala Phe His Asp Glu His 385 390 395 400	1200
ttt ggc ccc ttc ttc cga acc aac cag att ttt gtg aca gct aag aac Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Lys Asn 405 410 415	1248

agg tcc agc tac aag tac gac tcc ctg ctg cta ggg ccc aag aac ttc Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe 420 425 430	1296
agt ggg atc cta tcc ctg gac ttg ctg cag gag ctg ttg gag cta cag Ser Gly Ile Leu Ser Leu Asp Leu Leu Gln Glu Leu Leu Glu Leu Gln 435 440 445	1344
gag aga ctt cga cac ctg caa gtg tgg tcc cat gag gca cag cgc aac Glu Arg Leu Arg His Leu Gln Val Trp Ser His Glu Ala Gln Arg Asn 450 455 460	1392
atc tcc ctc cag gac atc tgc tat gct ccc ctc aac ccg cat aac acc Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro His Asn Thr 465 470 475 480	1440
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aac cac aca ctc ctg ctg ctc aca gcc aat cag act ctg aat ggc cag Asn His Thr Leu Leu Leu Thr Ala Asn Gln Thr Leu Asn Gly Gln 500 505 510	1536
acc tcc ctg gtg gac tgg aag gac cat ttc ctc tac tgt gcc aat gcc Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala 515 520 525	1584
cct ctc acg tac aaa gat ggc aca gcc ctg gcc ctg agc tgc ata gct Pro Leu Thr Tyr Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Ile Ala 530 535 540	1632
gac tac ggg gca cct gtc ttc ccc ttc ctt gct gtt ggg ggc tac caa Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln 545 550 555 560	1680
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gag gag gct ttc ttg aag gaa atg caa tcc ttc cag aga agc aca gct Glu Glu Ala Phe Leu Lys Glu Met Gln Ser Phe Gln Arg Ser Thr Ala 595 600 605	1824
gac aag ttc cag att gcg ttc tca gct gag cgt tct ctg gag gac gag Asp Lys Phe Gln Ile Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu 610 615 620	1872
atc aat cgc act acc atc cag gac ctg cct gtc ttt gcc atc agc tac Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Ile Ser Tyr 625 630 635 640	1920
ctt atc gtc ttc ctg tac atc tcc ctg gcc ctg ggc agc tac tcc aga Leu Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg	1968



Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val	
885 890 895	
ggg cct cca gtg tac ttt gac acc acc tca ggc tac aac ttt tcc acc	2736
Gly Pro Pro Val Tyr Phe Asp Thr Thr Ser Gly Tyr Asn Phe Ser Thr	
900 905 910	
gag gca ggc atg aac gcc att tgc tct agt gca ggc tgt gag agc ttc	2784
Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Glu Ser Phe	
915 920 925	
tcc cta acc cag aaa atc cag tat gcc agt gaa ttc cct aat cag tct	2832
Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asn Gln Ser	
930 935 940	
tat gtg gct att gct gca tcc tcc tgg gta gat gac ttc atc gac tgg	2880
Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp	
945 950 955 960	
ctg acc cca tcc tcc tcc tgc tgc cgc att tat acc cgt ggc ccc cat	2928
Leu Thr Pro Ser Ser Ser Cys Cys Arg Ile Tyr Thr Arg Gly Pro His	
965 970 975	
aaa gat gag ttc tgt ccc tca acg gat act tcc ttc aac tgt ctc aaa	2976
Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys	
980 985 990	
aac tgc atg aac cgc act ctg ggt ccc gtg aga ccc aca aca gaa cag	3024
Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr Thr Glu Gln	
995 1000 1005	
ttt cat aag tac ctg ccc tgg ttc ctg aat gat acg ccc aac atc	3069
Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Thr Pro Asn Ile	
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aga tgt cct aaa ggg ggc cta gca gcg tat aga acc tct gtg aat	3114
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ttg agc tca gat ggc cag att ata gcc tcc cag ttc atg gcc tac	3159
Leu Ser Ser Asp Gly Gln Ile Ile Ala Ser Gln Phe Met Ala Tyr	
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His Lys Pro Leu Arg Asn Ser Gln Asp Phe Thr Glu Ala Leu Arg	
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gca tcc cgg ttg cta gca gcc aac atc aca gct gaa cta cgg aag	3249
Ala Ser Arg Leu Leu Ala Ala Asn Ile Thr Ala Glu Leu Arg Lys	
1070 1075 1080	
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Val Pro Gly Thr Asp Pro Asn Phe Glu Val Phe Pro Tyr Thr Ile	
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Ser Asn Val Phe Tyr Gln Gln Tyr Leu Thr Val Leu Pro Glu Gly	
1100 1105 1110	



atc ttc act ctt gct ctc tgc ttc gtg ccc acc ttt gtg gtc tgc Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys 1115 1120 1125	3384
tac ctc cta ctg ggc ctg gac ata cgc tca ggc atc ctc aac ctg Tyr Leu Leu Leu Gly Leu Asp Ile Arg Ser Gly Ile Leu Asn Leu 1130 1135 1140	3429
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gtg tgg ggt atc agc tac aat gct gtg tcc ctc atc aac ctt gtc Val Trp Gly Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val 1160 1165 1170	3519
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tcc ttt gct gta agc acc aag cct acc cgg ctg gag aga gcc aaa Ser Phe Ala Val Ser Thr Lys Pro Thr Arg Leu Glu Arg Ala Lys 1190 1195 1200	3609
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atg acc aac ttc ccg ggc atc ctc atc ctg ggc ttt gct cag gcc Met Thr Asn Phe Pro Gly Ile Leu Ile Leu Gly Phe Ala Gln Ala 1220 1225 1230	3699
cag ctt atc cag att ttc ttc ttc cgc ctc aac ctc ctg atc acc Gln Leu Ile Gln Ile Phe Phe Phe Arg Leu Asn Leu Leu Ile Thr 1235 1240 1245	3744
ttg ctg ggt ctg cta cac ggc ctg gtc ttc ctg ccc gtt gtc ctc Leu Leu Gly Leu Leu His Gly Leu Val Phe Leu Pro Val Val Leu 1250 1255 1260	3789
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&lt;213&gt; Mus sp.

&lt;400&gt; 12

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Thr Phe Tyr Glu Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Gly Leu  
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Thr Ser Leu Ser Asn Ile Ser Cys Leu Ser Asn Thr Pro Ala Arg His  
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Val Thr Gly Asp His Leu Ala Leu Leu Gln Arg Val Cys Pro Arg Leu  
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Tyr Asn Gly Pro Asn Asp Thr Tyr Ala Cys Cys Ser Thr Lys Gln Leu  
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Val Ser Leu Asp Ser Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg  
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Cys Pro Ala Cys Ser Glu Asn Phe Val Ser Ile His Cys His Asn Thr  
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Cys Ser Pro Asp Gln Ser Leu Phe Ile Asn Val Thr Arg Val Val Gln  
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Arg Asp Pro Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr  
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Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Arg Val Arg  
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Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr

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Pro Gly Gln Ala Leu Ala Asp Gly Met Lys Pro Leu Asp Gly Lys Ile 225 230 235 240		
Thr Pro Cys Asn Glu Ser Gln Gly Glu Asp Ser Ala Ala Cys Ser Cys 245 250 255		
Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Pro Ala Leu 260 265 270		
Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile 275 280 285		
Ile Ile Phe Thr Ala Val Phe Val Leu Leu Ser Val Val Leu Val Tyr 290 295 300		
Leu Arg Val Ala Ser Asn Arg Asn Lys Asn Lys Thr Ala Gly Ser Gln 305 310 315 320		
Glu Ala Pro Asn Leu Pro Arg Lys Arg Arg Phe Ser Pro His Thr Val 325 330 335		
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Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ser Val 355 360 365		
Gly Leu Thr Phe Ile Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser 370 375 380		
Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ala Phe His Asp Glu His 385 390 395 400		
Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Lys Asn 405 410 415		

Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe  
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 Ser Gly Ile Leu Ser Leu Asp Leu Leu Gln Glu Leu Leu Glu Leu Gln  
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 Glu Arg Leu Arg His Leu Gln Val Trp Ser His Glu Ala Gln Arg Asn  
 450 455 460  
 Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro His Asn Thr  
 465 470 475 480  
 Ser Leu Thr Asp Cys Cys Val Asn Ser Leu Leu Gln Tyr Phe Gln Asn  
 485 490 495  
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 Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala  
 515 520 525  
 Pro Leu Thr Tyr Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Ile Ala  
 530 535 540  
 Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln  
 545 550 555 560  
 Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Ile  
 565 570 575  
 Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala His Ala Lys Leu Trp  
 580 585 590  
 Glu Glu Ala Phe Leu Lys Glu Met Gln Ser Phe Gln Arg Ser Thr Ala  
 595 600 605  
 Asp Lys Phe Gln Ile Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu  
 610 615 620  
 Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Ile Ser Tyr  
 625 630 635 640  
 Leu Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg  
 645 650 655

Trp Ser Arg Val Ala Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly  
660 665 670

Val Ala Val Val Leu Gly Ala Val Val Ala Ala Met Gly Phe Tyr Ser  
675 680 685

Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe  
690 695 700

Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu  
705 710 715 720

Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile  
725 730 735

Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu  
740 745 750

Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Ser Met Pro Ala  
755 760 765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Phe Asp Phe  
770 775 780

Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys  
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805 810 815

Asn Leu Pro Pro Pro Lys Gln Lys Glu Gly Leu Leu Leu Cys Phe Phe  
820 825 830

Arg Lys Ile Tyr Thr Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val  
835 840 845

Val Leu Leu Leu Phe Leu Val Leu Phe Gly Ala Asn Leu Tyr Leu Met  
850 855 860

Cys Asn Ile Ser Val Gly Leu Asp Gln Asp Leu Ala Leu Pro Lys Asp  
865 870 875 880

Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val  
885 890 895

Gly Pro Pro Val Tyr Phe Asp Thr Thr Ser Gly Tyr Asn Phe Ser Thr  
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Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Glu Ser Phe  
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Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asn Gln Ser  
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Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp  
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Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys  
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Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr Thr Glu Gln  
 995 1000 1005

Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Thr Pro Asn Ile  
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Arg Cys Pro Lys Gly Gly Leu Ala Ala Tyr Arg Thr Ser Val Asn  
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Leu Ser Ser Asp Gly Gln Ile Ile Ala Ser Gln Phe Met Ala Tyr  
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His Lys Pro Leu Arg Asn Ser Gln Asp Phe Thr Glu Ala Leu Arg  
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Val Pro Gly Thr Asp Pro Asn Phe Glu Val Phe Pro Tyr Thr Ile  
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Ser Asn Val Phe Tyr Gln Gln Tyr Leu Thr Val Leu Pro Glu Gly  
 1100 1105 1110

Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys

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Thr Ala Val Gly Met Ser 1175	Val Glu Phe Val Ser 1180	His Ile Thr Arg 1185
Ser Phe Ala Val Ser Thr 1190	Lys Pro Thr Arg Leu 1195	Glu Arg Ala Lys 1200
Asp Ala Thr Ile Phe Met 1205	Gly Ser Ala Val Phe 1210	Ala Gly Val Ala 1215
Met Thr Asn Phe Pro Gly 1220	Ile Leu Ile Leu Gly 1225	Phe Ala Gln Ala 1230
Gln Leu Ile Gln Ile Phe 1235	Phe Phe Arg Leu Asn 1240	Leu Leu Ile Thr 1245
Leu Leu Gly Leu Leu His 1250	Gly Leu Val Phe Leu 1255	Pro Val Val Leu 1260
Ser Tyr Leu Gly Pro Asp 1265	Val Asn Gln Ala Leu 1270	Val Leu Glu Glu 1275
Lys Leu Ala Thr Glu Ala 1280	Ala Met Val Ser Glu 1285	Pro Ser Cys Pro 1290
Gln Tyr Pro Phe Pro Ala 1295	Asp Ala Asn Thr Ser 1300	Asp Tyr Val Asn 1305
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Ala Glu Ala Gly Leu Arg Gly Trp Leu Leu Trp Ala Leu Leu Arg
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Leu Ala Gln Ser Glu Pro Tyr Thr Thr Ile His Gln Pro Gly Tyr Cys
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Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser Leu
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Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg Leu
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Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu Val
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Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg Cys
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Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr Cys
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&lt;211&gt; 1359

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 44

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Cys Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser  
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Leu Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg  
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Lys Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg  
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Leu Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu  
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Val Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg  
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Cys Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr  
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Cys Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln  
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Gln His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg  
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Val Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr  
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Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp  
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Gln Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu  
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Asp Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile

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Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro 450	Glu Ala Gln Arg Asn 455	
Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro 465	Leu Asn Pro Asp Asn Thr 470	
Ser Leu Tyr Asp Cys Cys Ile Asn Ser 485	Leu Leu Gln Tyr Phe Gln Asn 490	
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 675 680 685

Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe  
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Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile  
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Gly Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu  
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Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe  
770 775 780

Leu Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys  
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Arg Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln  
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850 855 860

Cys His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp  
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Ser Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val  
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Ser Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser  
930 935 940

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965 970 975

Asp Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn  
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 ccnggncarg gngarggny nytnytnngn tyyttycara argcntaygc nccnttyytn 2520  
 ytncaytgga thacnmngg ngtnngnytn ytnytnntty tngcnytnntt yggngtnwsn 2580  
 ytnaywsna tgtgycayt hwsngtnngn ytngaycarg arytnngcny nccnaargay 2640  
 wsntayytny tngaytayt ytnnttyytn aaymgntayt tygargtnng ngcncngtn 2700  
 taytgytna cnacnytnng ntayaaytyt wsnwsngarg cnggnatgaa ygcnahtgy 2760  
 wsnwsngcng gntgyaayaa ytywsntty acncaraara thcartaygc nacngartty 2820  
 ccngarcarw sntayytngc nathccngcn wsnwsntggg tngaygayt yathgaytg 2880  
 ytnacncnw snwsntgyt ymgnytnay athwsnggnc cnaayaarga yaarttytg 2940

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aayathaart gyccnaargg nggnytnngcn gcntaywsna cnwsngtnaa yytnacnwsn	3120
gayggncarg tnytnngcnws nmgnattyatg gcntaycaya arccnytnaa raaywsncar	3180
gaytayacng argcnytnmg ngcngcnmgn garytnngcn cnaayathac ngcngayytn	3240
mgnaargtnc cnggnacnga yccngcntty gargtnnttyc cntayacnat hacnaaygtn	3300
ttytaygarc artayytnac nathytnccn garggnytn tyatgytnws nytnngyytn	3360
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aayytnytnw snathgtnat gathytnngtn gayacngtn gnttyatggc nytnngggay	3480
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gcnaargarg cnacnathws natgggnwsn gcngtnttyg cngngngtngc natgacnaay	3660
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gargargcng tngcngcngt natggtngcn wsntgyccna aycayccnws nmngngtnwsn	3900
acngcngaya ayathtaygt naaycaywsn ttygarggnw snathaargg ngcngngcn	3960
athwsnaayt tyytnccnaa yaayggnmgn cartty	3996

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(54) Title: NPC1L1 (NPC3) AND METHODS OF IDENTIFYING LIGANDS THEREOF

(57) Abstract: The present invention provides human, rat and mouse NPC1L1 polypeptides and polynucleotides encoding the polypeptides. Methods for detecting ligands which bind to NPC1L1 and block intestinal cholesterol absorption are provided. Also included is a method of identifying ligands which bind to NPC1L1 using membranes derived from brush border membrane preparations. Compounds that bind to NPC1L1 can be used for inhibiting intestinal cholesterol absorption in a subject.

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## INTERNATIONAL SEARCH REPORT

International application No.

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<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(7) : G01N 33/53, 33/532, 33/534; C12Q 1/60 US CL : 435/7.1, 7.93, 11; 436/544, 545 According to International Patent Classification (IPC) or to both national classification and IPC											
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/7.1, 7.93, 11; 436/544, 545 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Continuation Sheet											
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <table border="1"> <thead> <tr> <th>Category *</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>A</td> <td>US 2004/0132058 A1 (Altmann et al.) 08 July 2004 (08.07.2004), especially paragraph [0011].</td> <td>1, 7, 8, 10-13</td> </tr> <tr> <td>A</td> <td>US 2004/0161838 A1 (Altmann et al.) 19 August 2004 (19.08.2004), especially paragraph [0011].</td> <td>1, 7, 8 and 10-13</td> </tr> </tbody> </table>			Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	A	US 2004/0132058 A1 (Altmann et al.) 08 July 2004 (08.07.2004), especially paragraph [0011].	1, 7, 8, 10-13	A	US 2004/0161838 A1 (Altmann et al.) 19 August 2004 (19.08.2004), especially paragraph [0011].	1, 7, 8 and 10-13
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US05/01469

Continuation of B. FIELDS SEARCHED Item 3:

APS, CAPLUS, STN search, East search.

Search terms : NPC3, NP1C1L1, azetidinone, ezetimibe, label, ligand, agonist, antagonist, detection, identification.